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ROLE OF MATRIX METALLOPROTEINASE-2 IN OXIDATIVELY STRESSED HEARTS

BY

WENJIE WANG

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

DEPARTMENT OF PHARMACOLOGY

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ABSTRACT

Matrix metalloproteinases (MMPs) are best known for their roles in the long term remodeling of the extracellular matrix. Our group recently has demonstrated that MMP-2 is involved in acute myocardial ischemia-reperfusion injury. I studied the regulation and molecular action of MMP-2 in the oxidatively stressed heart. In such hearts, stressed either by the infusion of peroxynitrite or the NO (nitric oxide) synthase inhibitor, N^G-nitro-L-arginine methyl ester, I found an increase in MMP-2 activity in the perfusate. An inhibitor of MMPs, PD-166793, protected the heart from peroxynitrite-induced injury. In another form of oxidative stress to the heart, ischemia and reperfusion, I found an imbalance between tissue inhibitors of metalloproteinases (TIMPs) and MMPs, resulting in an enhanced gelatinolytic activity. I also demonstrated that TIMP-4, the most abundant form of TIMP in the heart, is associated with sarcomeres in the normal heart but its content is reduced in ischemic-reperfused hearts.

The molecular mechanism of MMP-2-induced myocardial ischemia-reperfusion injury is unknown. The degradation of contractile proteins such as troponin I is suggested to be part of the pathophysiology of ischemia-reperfusion injury. I studied the role of MMP-2 in the proteolysis of troponin. MMP-2 was able to proteolyze troponin I and T but not troponin C in vitro. Only troponin I was susceptible to degradation by MMP-2 in the native troponin complex. In isolated perfused hearts, inhibitors of MMPs (o-phenanthroline and doxycycline) not only improved the recovery of mechanical function but also prevented the loss of troponin I. Immunoelectron microscopy revealed the colocalization of MMP-2 with sarcomeres. Immunoprecipitation and confocal microscopy studies demonstrated the association of MMP-2 and troponin I within cardiac

myocytes. These data present novel, clear-cut evidence of the intracellular action of MMP-2 in the cardiac myocyte during ischemia-reperfusion injury.

In summary, I found a role of MMPs in the oxidatively stressed heart induced either by peroxynitrite infusion, by removal of basal NO generation or by subjecting the heart to ischemia-reperfusion. I also discovered a novel intracellular action of MMP-2 on troponin I within the cardiac myocyte. These data offer new insight in the pathology of oxidative stress injury to the heart and provide therapeutic strategy.

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God bless all of you

Dedicated to

My lovely wife

Ning Kang

TABLE OF CONTENTS

ABSTRACT i

ACKNOWLEDGEMENTS iii

DEDICATION iv

TABLE OF CONTENTS v

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF PUBLICATIONS xiii

ABBREVIATIONS xv

CHAPTER I INTRODUCTION 1

1.1 Matrix metalloproteinases 2

- 1.1.1 History and nomenclature
- 1.1.2 Regulation of MMP activity
- 1.1.3 Inhibitors of MMPs
- 1.1.4 Extracellular matrix related roles of MMPs
- 1.1.5 Novel substrates and biological effects of MMPs

1.2 Nitric oxide and peroxynitrite 17

- 1.2.1 Nitric oxide physiology
- 1.2.2 Peroxynitrite pathophysiology
- 1.2.3 Nitric oxide, peroxynitrite and myocardial ischemia-reperfusion injury
- 1.2.4 Nitric oxide as an anti-oxidant species
- 1.2.5 Nitric oxide and MMPs
- 1.2.6 Oxidants and MMPs

1.3 Myocardial ischemia-reperfusion (stunning) injury 24

- 1.3.1 History and definition
- 1.3.2 Reactive oxygen species and myocardial ischemia-reperfusion injury
- 1.3.3 Contractile proteins and myocardial ischemia-reperfusion injury
- 1.3.4 Isolated heart perfusion as a myocardial ischemia-reperfusion model

1.4 Objectives and hypothesis 31

1.5 References 34

CHAPTER II MATERIALS AND METHODS 68

2.1 Materials 69
2.2 Heart preparation and perfusion 70
2.3 Perfusion protocol 71
2.4 Concentration of proteins in the coronary effluent 74
2.5 Preparation of heart extracts 74
2.6 Gelatin zymography 75
2.7 Conventional electron microscopy 75
2.8 Electron microscopy with immunogold labeling 76
2.9 Isolation of thin myofilaments 77
2.10 Statistical analysis 78
2.11 References 79

CHAPTER III PEROXYNITRITE-INDUCED MYOCARDIAL INJURY IS MEDIATED THROUGH MATRIX METALLOPROTEINASE-2 80

3.1 Introduction 81
3.2 Materials and methods 82
3.3 Results 84
3.4 Discussion 87
3.5 References 98

CHAPTER IV INHIBITION OF NITRIC OXIDE SYNTHASES IN THE HEART ENHANCES MATRIX METALLOPROTEINASE-2 RELEASE VIA OXIDATIVE STRESS 105

4.1 Introduction 106
4.2 Materials and methods 107
4.3 Results 108
4.4 Discussion 110
4.5 References 118

CHAPTER V THE IMBALANCE BETWEEN TISSUE INHIBITOR OF METALLOPROTEINASE-4 AND MATRIX METALLOPROTEINASES DURING ACUTE MYOCARDIAL ISCHEMIA-REPERFUSION INJURY 123

5.1 **Introduction** 124
5.2 **Materials and methods** 125
5.3 **Results** 127
5.4 **Discussion** 130
5.5 **References** 140

CHAPTER VI INTRACELLULAR ACTION OF MATRIX METALLOPROTEINASE-2 ACCOUNTS FOR ACUTE MYOCARDIAL ISCHEMIA-REPERFUSION INJURY 144

6.1 **Introduction** 145
6.2 **Materials and methods** 146
6.3 **Results** 149
6.4 **Discussion** 153
6.5 **References** 165

CHAPTER VII CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS 170

7.1 **Conclusions** 171
7.2 **Limitations** 177
7.3 **Future directions** 180
7.4 **References** 183

LIST OF TABLES

* indicates data generated by myself

Table 1.1 Matrix metalloproteinases. 3

***Table 3.1** Effects of decomposed $ONOO^-$ or $ONOO^-$ on heart rate and left ventricular developed pressure. 92

***Table 4.1** Effects of treatments on heart rate, left ventricular developed pressure and coronary perfusion pressure in isolated perfused rat hearts. 113

LIST OF FIGURES

* indicates data generated by myself

Figure 1.1 Domain structure of MMP-2. 6

Figure 1.2 Activation of MMPs. 8

Figure 1.3 Cardiac contractile apparatus. 27

* **Figure 3.1** Effects of peroxynitrite or decomposed peroxynitrite on coronary perfusion pressure and mechanical function measured as the rate-pressure product in isolated perfused rat hearts. 93

* **Figure 3.2** Time-dependent effects of peroxynitrite on the release of gelatinolytic activity into the coronary effluent. 94

* **Figure 3.3** Effects of 300 μM glutathione (GSH) infusion on changes in 72-kDa gelatinolytic activity in coronary effluent from hearts subjected to 80 μM peroxynitrite and cardiac mechanical function. 95

* **Figure 3.4** Effect of 2 μM PD-166793 on MMP-2 release into the coronary effluent stimulated by 80 μM peroxynitrite infusion and on mechanical function. 96

* **Figure 3.5** Effects of 3 μM SNAP or vehicle on coronary perfusion pressure, mechanical function and MMP-2 release into coronary effluent. 97

* **Figure 4.1** Effects of L-NAME on coronary perfusion pressure and MMP-2 release into the coronary effluent from

isolated perfused rat hearts. 114

* **Figure 4.2** Effects of co-infusion of SNAP with L-NAME on coronary perfusion pressure and MMP-2 release into coronary effluent. 115

* **Figure 4.3** Effect of U46619 infusion on coronary perfusion pressure and MMP-2 release into coronary effluent, in comparison to L-NAME. 116

* **Figure 4.4** Effect of treatments on lipid hydroperoxide level in myocardium. 117

* **Figure 5.1** Cardiac mechanical function of isolated rat hearts subjected to 20 min ischemia and 30 min reperfusion. 134

Figure 5.2 Reverse zymography and Western blot analysis of coronary effluent samples from hearts subjected to 20 min ischemia and 30 min reperfusion. 135
(Reverse zymography was done with the help of C. Schulze)

* **Figure 5.3** Cardiac mechanical function and TIMP-4 release from hearts subjected to different durations of ischemia. 136

* **Figure 5.4** Immunogold electron micrographs of control hearts aerobically perfused for 75 min and hearts subjected to 20 min ischemia followed by 30 min reperfusion. 137

Figure 5.5 Identification of TIMP-4 in thin myofilament preparations from control hearts aerobically perfused for 75 min, but not from hearts subjected to 20 min ischemia followed by 30 min reperfusion.

(a and c was done with the help of C. Schulze) 138

***Figure 5.6** In situ zymograms from control hearts aerobically perfused for 75 min or from hearts subjected to 20 min followed by 30 min reperfusion. 139

Figure 6.1 SDS -PAGE silver stain showing in vitro degradation of troponin I and T by MMP-2. *(with the help of C. Schulze and G. Sawicki)* 157

Figure 6.2 SDS-PAGE silver stain showing in vitro degradation of TnI within the troponin complex by MMP-2. *(Western blot analysis was done by C. Schulze)* 158

Figure 6.3 Cardiac mechanical function of isolated hearts subjected to ischemia and reperfusion and its relation to myocardial TnI content. *(with the help of C. Schulze)* 159

Figure 6.4 Effect of infusion of MMP-2 into isolated hearts on recovery of cardiac mechanical function after 15 min ischemia and 30 min reperfusion and densitometric analysis of myocardial TnI content as determined by western blot. *(Western blot analysis was done by C. Schulze)* 160

Figure 6.5 Association of MMP-2 with TnI after ischemia-reperfusion. *(with the help of C. Schulze)* 161

Figure 6.6 Intracellular localization of MMP-2 within thin myofilaments. (*with the help of C. Schulze*) 162

Figure 6.7 Multitracking scanning using barrier filters up to 20 sections (0.4 μm each) clearly showed intracellular localization of MMP-2 within cardiac myocytes. *(with the help of W. L. Suarez-Pinzon)* 163

Figure 6.8 Co-localization of MMP-2 with TnI using immunofluorescent confocal microscopy. *(with the help of W. L. Suarez-Pinzon)* 164

Figure 7.1 Model of MMP-2 induced injury 175

Figure 7.2 MMP-2 and TIMP-4 in ischemia and reperfusion 176

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A) Papers:

* Equal first author contribution

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5. **Wang, W.,** Sawicka, J., Schulz, R. Inhibition of NO synthases increases matrix metalloproteinase-2 release in the heart. *Br J Pharmacol*, under revision.
6. Schulze, C.,* **Wang, W.,*** Sawicki, G., Schulz, R. The imbalance between tissue inhibitor of metalloproteianse-4 and matrix metalloproteinase during acute myocardial ischemic-reperfusion injury. Submitted to *Circulation.*
7. Lalu, M., **Wang, W.,** Schulz, R. Peroxynitrite in acute myocardial ischemia-reperfusion injury. *Heart Fail Rev* (invited review), in press.

B) Peer reviewed abstracts:

1. **Wang, W.,** Sawicka J, Sawicki G, Schulze C, Schulz, R. Matrix metalloproteinase-2 is responsible for troponin T degradation in myocardial ischemia-reperfusion injury. Submitted to the 75th Annual meeting of the American Heart Association.
2. **Wang, W.,** Schulze C, Suarez-Pinzon W, Sawicki G, Schulz, R. Ischemia-reperfusion reduces tissue inhibitor of metalloproteinase-4 (TIMP-4) in cardiac myocyte. Submitted to the 75th Annual meeting of the American Heart Association.
3. Sawicki G, Leon H, **Wang, W.,** Sawicka J, Schulze C, Schulz, R. Proteomic analysis of ischemic-reperfused hearts functionally protected with matrix metalloproteinases inhibitor. Submitted to the 75th Annual meeting of the American Heart Association.

4. Leon H, Sawicki G, **Wang, W.,** Sawicka J, Schulze C, Schulz, R. Time-dependent protein release during reperfusion following myocardial ischemia: A proteomics approach. Submitted to the 75th Annual meeting of the American Heart Association.
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ABBREVIATIONS

ANOVA	analysis of variance
ADP	adenosine diphosphate
AP-1	Activating protein-1
APMA	4-aminophenylmercuric acetate
cGMP	cyclic 3',5'-guanosine monophosphate
DAPI	4',6-diamidino-2-phenyl indole
DMSO	dimethyl sulfoxide
EDTA	ethylene-diaminetetra-acetic acid
eNOS	endothelial nitric oxide synthase
Erk	extracellular signal-regulated kinase
ET-1	endothelin-1
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GSH	Glutathione
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
I/R	ischemia-repefusion
L-NAME	N^G-nitro-L-arginine ester
L-NMMA	N^G-monomethyl L-arginine
LVDP	left ventricular developed pressure
MMPs	matrix metalloproteinases
MT-MMP	membrane type matrix metalloproteinase

NADPH	nicotinamide adenosine dinucleotide phosphate
NF-κB	nuclear factor-κB
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
$ONOO^-$	peroxynitrite
$O_2^{\bullet -}$	superoxide
$OH^{\bullet}$	hydroxyl radical
PBS	phosphate buffered saline
PEA3	polyomavirus enhancer A 3
PNT	o-phenanthroline
RPP	rate pressure product
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIN-1	1,3-morpholinosydnonimine-HCl
SNAP	S-nitroso-N-acetylpenicillamine
TIMPs	tissue inhibitors of metalloproteinases
TnI	troponin I
TnT	troponin T
TnC	troponin C
TNF-α	tumor necrosis factor-α
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER I

INTRODUCTION

1.1 Matrix metalloproteinases

1.1.1 History and nomenclature

In 1962, Gross and Lapiere first found a collagenolytic activity in amphibian tissue and coined the name "collagenase" [1]. It was later named matrix metalloproteinase-1 (MMP-1).[2] The MMP family is still growing with at least 28 members to date.[3] Table 1.1 lists the characteristics of some mammalian MMPs (Table 1.1). MMPs are zinc-containing endopeptidases that can act synergistically to degrade the components of extracellular matrix.[4] Based on their in vitro substrate preference and structural homology, MMPs are categorized into five groups: collagenases, gelatinases, stromelysins, membrane-type MMPs (MT-MMPs) and miscellaneous MMPs.[2] Each group has distinct features but also share common properties with other groups. For example, MT-MMPs are different from soluble MMPs because they are membrane-bound and are important in the activation of soluble MMPs, in particular MMP-2.[5]

Gelatinases (gelatinase A = 64-kDa type IV collagenase = MMP-2, gelatinase B = 84-kDa type IV collagenase = MMP-9), are of particular interest due to their unique characteristics. For example, MMP-2 is expressed constitutively at a higher level than any other MMP[6], while MMP-9 is induced easily by pro-inflammatory cytokines or oncogene products.[7] Moreover, gelatinases have been recognized to play an important role in a number of physiological and pathological conditions such as embryogenesis, bone repair, wound healing and angiogenesis.[4]

Table 1.1 **Matrix metalloproteinases**

Name	MMP number	M_r latent/active* (kDa)
Collagenases		
Collagenase 1	MMP-1	52/42
Collagenase 2	MMP-8	85/64
Collagenase 3	MMP-13	52/42
Collagenase 4	MMP-18	53/42
Gelatinases		
Gelatinase A	MMP-2	72/64
Gelatinase B	MMP-9	92/84
Stromelysins		
Stromelysin 1	MMP-3	57/45
Stromelysin 2	MMP-10	54/44
Stromelysin 3	MMP-11	64/46
Membrane-type MMPs		
MT1-MMP	MMP-14	66/54
MT2-MMP	MMP-15	72/60
MT3-MMP	MMP-16	64/53
MT4-MMP	MMP-17	57/53
Others		
Matrilysin	MMP-7	28/19
Metalloelastase	MMP-12	54/22
(No trivial name)	MMP-19	54/45
Enamelysin	MMP-20	54/22

*Note: some latent forms of MMPs have been shown to be activated by oxidants without proteolytic cleavage[8,9]. Mr, molecular weight. (Adapted from Parks WC, Mecham RP: Matrix metalloproteinases, San Diego: Academic Press; 1998; p. 6)

1.1.2 Regulation of matrix metalloproteinase activity

1.1.2.1 Transcriptional and translational regulation

MMP-2 and MMP-9 genes have been cloned.[10] The MMP-2 gene has the characteristics of a "housekeeping" gene due to lack of a TATA box and binding sites for PEA3, AP-1 and transforming growth factor-β inhibitory element in the promoter region.[11] As a result, the expression of MMP-2 is ubiquitous and the regulation of its activity relies more on post-translational events.[6]

Compared with the MMP-2 gene, the human MMP-9 gene is inducible by pro-inflammatory cytokines and other transcriptional regulators including tumor necrosis factor-α and interleukin-1, growth factors, oncogene products, and endotoxin.[12-14] These are supported by the gene structure of MMP-9 which includes a TATA box and an AP-1 binding site.[15] Sato *et al.* found that the NF-κB and SP-1 binding site-like sequences were also essential for 12-O-tetradecanoyl-phorbol-13-acetate- and tumor necrosis factor-α-mediated induction of MMP-9, suggesting that its expression was regulated by a combination of AP-1 proteins with NF-κB-like and SP-1-like factors.[16] In contrast, a sequence for the transforming growth factor-β inhibitory element has also been identified in the MMP-9 gene.[15] In fact, transforming growth factor-β, as well as interferon-β, interferon-γ, progesterone and corticosteroids, were found to be capable of suppressing the expression of other MMPs.[7]

A number of MMPs have been identified in the heart including MMP-1, MMP-2, MMP-7, MMP-9 and MMP-13[17-20]. MMP-2 is expressed in endothelium[19], endomyocardial-derived endothelial cells[21], vascular smooth muscle cells[22,23], cardiac myocytes[24] and connective tissue[25]. Upon stimulation as mentioned above and ischemia-reperfusion injury[24], MMP-9 is expressed in cardiac myocytes, endothelial cells, smooth muscle cells and macrophages[26].

1.1.2.2 Post-translational regulation

MMP-2 and MMP-9 share a similar protein structure.[27,28] From the amino terminal, there are signal peptide, propeptide, catalytic and fibronectin-like domains and a carboxyl-terminal hemopexin/vitronectin-like domain (Figure 1.1). The signal peptide domain serves as a signal sequence guiding the transport of enzymes into the endoplasmic reticulum. The propeptide domain acts as a "cap" to help maintain the proenzyme in an inactive state. A highly conserved sequence PRCGVPDV has been identified in this part. In the catalytic domain, three histidine residues in a highly conserved sequence HEXXHXXGXXH are found in all MMPs and other zinc-dependent endopeptidases.[29-31] These histidine residues coordinate and stabilize the zinc ion within the catalytic domain.[29] The cysteine residue in the PRCGVPDV sequence interacts with the zinc active site in the catalytic domain keeping the proenzyme latent.[32,33] This complex keeps H_2O, which is critical for catalysis, away from zinc. The "on" and "off" of this cysteine and zinc determines whether the activity of enzyme is "on" or "off". This is the process first named by Van Wart and Birkedal-Henson as the "cysteine switch" hypothesis.[32] A fibronectin-like domain which consists of three repeats, each 58 or 59 amino acids in length and resembling collagen-binding type II units in fibronectin, is a unique feature of both MMP-2 and MMP-9.[30] This domain equips MMP-2 and MMP-9 with the ability to bind gelatin and serves as a substrate docking socket.[30] A hemopexin/vitronectin-like carboxyl terminal domain facilitates the interaction between MMPs and tissue inhibitors of metalloproteinases (TIMPs).[34] Between the catalytic domain and hemopexin/vitronectin-like domain is a variable hinge domain that may participate in protein folding.

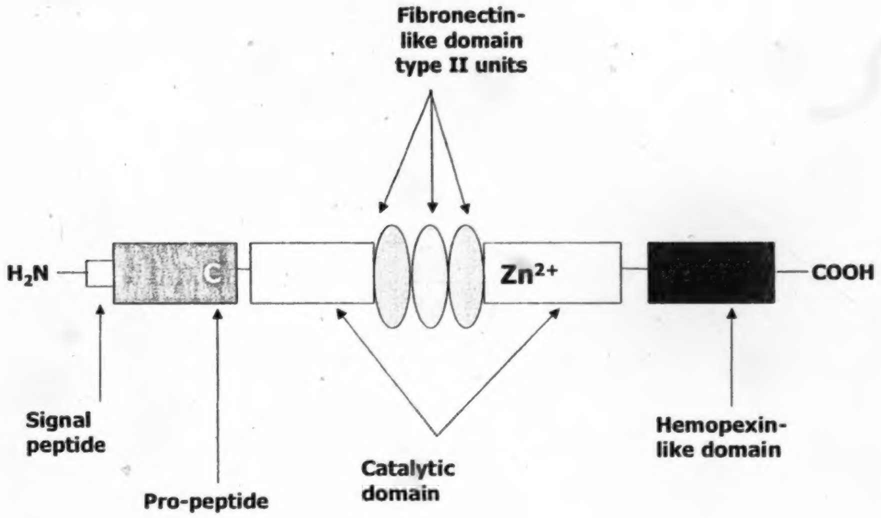
Fibronectin-like domain type II units
H₂N
C
Zn²⁺
COOH
Signal peptide
Pro-peptide
Catalytic domain
Hemopexin-like domain

Figure 1.1 Domain structure of MMP-2. C, conserved cysteine. (Adapted from Nagase H. Activation mechanism of matrix metalloproteinases. Biol Chem 1997; 378:151-160)

The activation process is important in the regulation of MMP activity.[8] The milestone of the activation of MMPs is the disruption of the cysteine-zinc bond which can be achieved by two distinctive pathways in vitro. Proteases such as plasminogen, trypsin, kallikrein and MMPs can activate MMPs by proteolysis of the propeptide domain.[35,36] The active MMP then has a lower molecular weight, normally by 8-10 kDa.

The activation of MMPs can also be achieved by organomercurials, thiols, oxidants and chaotropes (e.g. 3M NaSCN).[8] In this process, a protein conformational change occurs which removes cysteine from zinc. Further cleavage of the propeptide by MMPs or other proteases is not necessary.[9] Figure 1.2 illustrates the activation procedures of MMPs.

How the activation of MMP-2 happens in the cell is not yet conclusively known. The intensive research in this area has accumulated abundant evidence to piece the puzzle together. The process is generally believed to take place on the plasma membrane.[37-40] Both proMMP-2 and TIMP-2 were found to accumulate in the invadopia, a specialized membrane extension, by an activator localized to the cell surface.[6,38] Sato *et al.* later cloned and named this activator MT-MMP.[41] The tri-molecule complex has been identified on the surface of different cells.[42-44] Other components have also been shown in the cell surface interaction with proMMP-2, such as integrin $\alpha_v\beta_3$.[45] TIMP-2 plays a biphasic role in the activation of proMMP-2. At high concentrations, it blocks the activation of proMMP-2, but a stoichiometric amount is required for the formation of the activation complex.[6,44]

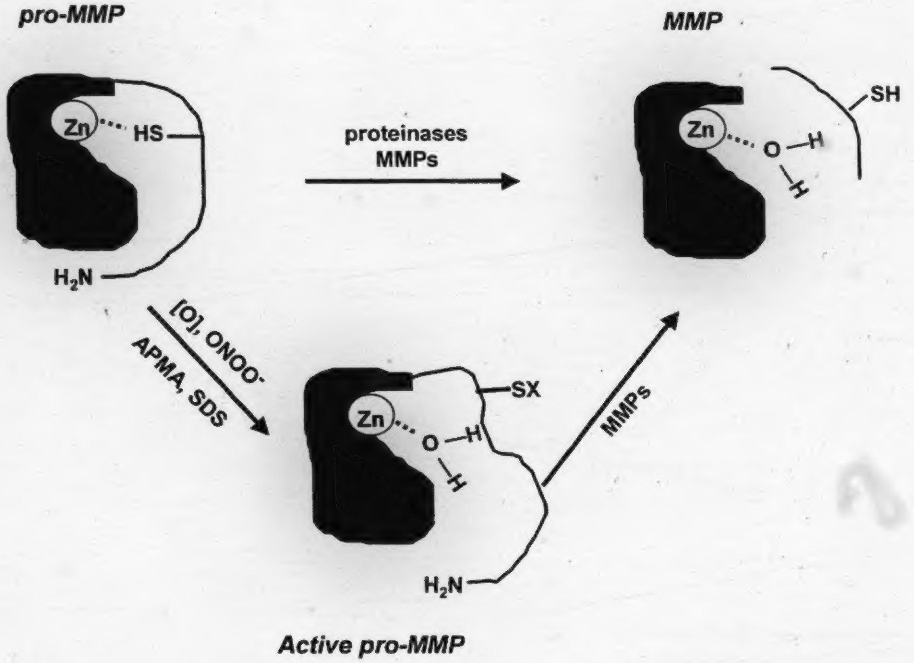
pro-MMP
Zn
HS
H_2N
proteinases
MMPs
MMP
Zn
O—H
H
SH
[O], ONOO⁻
APMA, SDS
Zn
O—H
H
SX
H_2N
MMPs
Active pro-MMP

Figure 1.2 The activation of MMPs. [O]: Oxidants, $ONOO^-$: Peroxynitrite, APMA: 4-aminophenylmercuric acetate; SDS: Sodium dodecyl sulfate; SH: reduced sulfhydryl group; SX: oxidized sulfhydryl group. (Adapted from Parks WC, Mecham RP: Matrix metalloproteinases, San Diego: Academic Press; 1998; p.87).

1.1.3 Inhibitors of matrix metalloproteinases

1.1.3.1 Natural inhibitors

Although α_2-macroglobulin functions as a clearance protein for the removal of MMPs from the circulation by binding to them,[46] TIMPs are the more important endogenous inhibitors of MMPs in a manner of reducing their catalytic activity. Four TIMP isoforms currently have been cloned and characterized (TIMP-1, -2, -3 and -4).[47] They all possess 12 cysteine residues in conserved regions forming six disulfide bonds, which make up "disulfide knots" in the tertiary structure. The region surrounding the second and third "disulfide knots" (Cys^{13}-Cys^{124}, $\text{Cys}\ ^{127}$-Cys^{174}) in human TIMP-1 plays a major role in the inhibition of fibroblast-type collagenase.[48] The amino terminus is a necessary component for TIMP-2 to inhibit MMP activity.[49] C-terminal domains of TIMPs are important in binding with MMPs[50]. All TIMPs inhibit MMPs by forming a noncovalent complex in a 1:1 molar ratio. TIMPs show different abilities to inhibit MMPs. For example, TIMP-1 strongly binds to pro-MMP-9 whereas TIMP-2 preferentially binds to pro-MMP-2[51].

The pattern of TIMP expression is variable. TIMP-1 is expressed at a low level in normal conditions and is induced in fibroblasts[52,53], lung[54], brain astrocytes[55] and connective tissue[56], and other cells, upon stimulation with basic fibroblast growth factor, platelet-derived growth factor, interleukin-6 or hypoxic injury. TIMP-2 is widely expressed under physiological conditions[47]. TIMP-3 is inducible and is the only TIMP tightly bound to extracellular matrix [57]. Notably, TIMP-3 mRNA is found in the heart [57]. TIMP-4 was first reported to be expressed selectively in human heart and undetectable in other organs[58] but was later found to be expressed in a much broader range of tissues including brain, ovary, smooth muscle cells, skeletal muscle, and skin in both mouse[59] and rat.[60]

The best known role of TIMPs is to inhibit MMP activity. Paradoxically, TIMP-2 is also important in the pericellular activation of proMMP-2[7]. In addition, TIMP-1 and TIMP-2 are also known as erythroid potentiating activator, which stimulates the growth of early erythroid progenitor cells and demonstrates growth-promoting activity on a variety of cultured cell lines.[61,62] The role of TIMP-4 is not yet clear, although it is abundantly expressed in the myocardium[58,59]. Interestingly, Tummalapalli *et al.* found that TIMP-4 is highly expressed within normal cardiac fibroblasts and is involved in the regulation of normal cell phenotype.[63] Moreover, TIMP-4, unlike TIMP-2, does not promote but actually inhibits proMMP-2 activation, indicating a unique biological action of TIMP-4.[64,65] The physiological role of TIMP-4 requires further investigation.

Studies with transgenic animals have provided unique information on the individual roles of TIMPs and MMPs in physiology and pathophysiology. In TIMP-1-deficient mice, their spontaneous development of left ventricular enlargement suggests a critical role of TIMP-1 in the maintenance of normal myocardial structure[66]. After vascular injury, impaired wound healing was seen in TIMP-1 knockout mice[67]. TIMP-3-deficient mice showed early mortality due to spontaneous air space enlargement in the lung[68]. An imbalance between TIMPs and MMPs which favors extracellular matrix destruction, was suggested in these transgenic animals. Indeed, MMP-9 knockout mice demonstrated reduced left ventricular enlargement after myocardial infarction[69]. However, upregulation of MMP-3 and MMP-13 was seen in the MMP-9 knockout mice, indicating the redundant nature of MMPs, which may underestimate the significance of individual MMPs or TIMPs[69]. Caution should, therefore, be used when interpreting results from transgenic animals.

In many cardiac pathologies in which long-term remodeling processes occur, the imbalance between TIMPs and MMPs has been demonstrated. These include myocardial infarction,[70,71] heart failure[72,73] and dilated cardiomyopathy.[74] A reduction of TIMPs and an enhancement of MMPs lead to an increased tissue net MMP activity. Very recently, Mayers *et al.* found a reduced level of TIMP-4 in right atrium in patients undergoing cardiopulmonary bypass and an increase in MMP-9 activity.[75] This is the first report in the literature demonstrating that the level of TIMP-4 changes as a result of acute treatment. However, the changes of TIMPs, in particular TIMP-4 in the heart, during acute myocardial ischemia-reperfusion injury, are not yet clear.

1.1.3.2 Synthetic inhibitors

Synthetic inhibitors of matrix metalloproteinase are essentially divalent ion chelators. Hydroxamate mimetics are designed based on the Ile-Ala-Gly and Leu-Leu-Ala sequences on the right-hand side of the cleavage site in the collagen molecule[76]. Some of these compounds such as batimastat and marimastat have already been tested in clinical trials of cancer chemotherapy and rheumatoid arthritis. Because of their non-selectivity between individual MMPs, their adverse effects including musculoskeletal pain seen in patients taken marimastat, and no beneficial effect on survival, these compounds are no longer pursued for cancer chemotherapy[77-79]. Other non-peptide and low molecular weight MMP inhibitors have also been used such as o-phenanthroline[80].

anti-collagenase activity of tetracycline antibiotics discovered by Golub *et al.*, unrelated to their antibacterial action, deserves further attention due to their low toxicity and wide usage as antibiotics[81].

Doxycycline is one of the tetracyclines with the most potent anti-MMP action and has been the best studied tetracycline. Doxycycline was shown to be able to inhibit gelatinases[82] and in particular MMP-2.[83] Doxycycline inhibits MMP activity by both direct and indirect mechanisms[84]. Doxycycline was suggested to be able to chelate Ca^{2+} and Zn^{2+}.[85] Indeed, a Ca^{2+} and Zn^{2+} binding moiety has been identified in the doxycycline molecule. Based on this information, chemically modified tetracyclines have been engineered specifically to reduce their antimicrobial action but retain MMP inhibitory activity[86]. In addition, tetracyclines have also been suggested to be able to inhibit the oxidative activation of proMMPs, protect α_1-protease inhibitor from degradation and inhibit cytokine production, just to name a few possible additional effects[84].

1.1.4 Extracellular matrix-related roles of MMPs

1.1.4.1 Extracellular matrix substrates of MMP-2

Although named "gelatinase A" or "type IV collagenase", MMP-2 has a very broad substrate spectrum in vitro. MMP-2 is able to cleave gelatin, type I, IV, V, VII, and X collagens,[87-89] elastin,[90] vitronectin[91] and laminin-5.[92] MMP-2 cleaves gelatin at a number of dipeptide bonds such as Gly-Val, Gly-Leu, Gly-Glu, Gly-Ile, Gly-Asn and Gly-Ser, resulting in smaller peptide fragments.[93,94] In soluble, triple helical type I collagen, the MMP-2 cleavage bonds of Gly-Ile/Leu are located exactly at three quarters from the N-terminus and one quarter from the C-terminus producing fragments typically found by the cleavage of MMP-1.[95] Interestingly, MMP-2 is only able to degrade solubilized type IV collagen but not native full-length type IV collagen.[96,97] These observations indicate that the names "gelatinase A" or "type IV collagenase" are misnomers and underestimate the proteolytic spectrum of MMP-2.

Our knowledge about the in vivo substrates of MMP-2 is even sparser. The above mentioned extracellular matrix components are only assumed to be in vivo substrates of MMP-2. Beside these, little is known about the exact substrates of MMP-2 in vivo. There are some reports that MMP-2 is able to cleave amyloid protein precursor, and therefore it possesses β-secretase, but not α-secretase activity.[98,99] Many new in vivo substrates of MMP-2 will need to be explored in the near future.

1.1.4.2 Physiological and pathophysiological roles of MMPs

Being able to degrade many components of extracellular matrix, MMP-2 has been shown to be involved in many physiological and pathological processes. The list of physiological conditions includes morphogenesis, cartilage and bone repair, wound healing, angiogenesis and cell migration.[4] The MMPs have also been implicated in many pathologies such as cancer, inflammation, cardiovascular diseases, neurological diseases, liver fibrosis and fibrotic lung diseases, just to name a few[4]. It is believed that MMPs not only function by removal of the extracellular matrix during tissue resorption and progression of diseases but also may alter the biological function of extracellular matrix molecules by specific cleavage.[100] For example, the fragment of laminin 5 cleaved by MMP-2 is able to induce migration of normal breast epithelial cells.[92] Mice lacking

MMP-7 or MMP-11 reveal that these MMPs are important in the initiation or growth of primary and metastatic tumors.[101,102]

In cardiovascular diseases such as myocardial infarction[71], heart failure[103,104], dilated cardiomyopathy,[105] atherosclerosis[106] and aortic aneurysm[107], the role of MMPs has been established. A dynamic turnover of extracellular matrix is suggested to occur in these pathologies. For example, a rapid decrease (in hours) in collagen content was shown in infarcted myocardium.[108] A number of MMPs such as MMP-1, MMP-2, MMP-3 and MMP-9 are upregulated in rat[70,109], pig[110] and human[111] hearts after myocardial infarction. Consequently, inhibition of MMP activity attenuated the enlargement of left ventricle early after myocardial infarction.[112] More recently, transgenic animal studies reveal a definitive role of MMP-9 in infarct healing[113] and left ventricular dilation[69] after myocardial infarction. MMP-9 deficient mice showed a retarded healing and attenuated left ventricular enlargement. Although these animal studies strongly support the critical role of MMPs in these cardiac pathologies, inhibition of MMPs as a therapeutic approach has not been attempted. Many fundamental questions such as the timing of MMP inhibitor administration, selective versus broad-range inhibitors, and possible side effects of MMP inhibitors, etc. require to be clarified before any clinical trial could be considered.

1.1.5 Novel substrates and biological effects of MMP-2

Recently, MMPs, in particular MMP-2, are reported to have some novel effects unrelated to their actions on the extracellular matrix. Sawicki *et al.* presented a seminal paper showing that MMP-2 is released from platelets during activation with collagen and subsequent aggregation. This release is involved in the regulation of platelet aggregation because inhibition of MMPs prevented platelet aggregation.[114] Kazes *et al.* confirmed the finding later.[115] In contrast, MMP-9, despite its structural and known substrate similarity with MMP-2, is an inhibitor of platelet aggregation.[116] MMP-2/MMP-9 are important regulators of platelet function in addition to the NO/cGMP, thromboxane A_2 and ADP pathways.

Regulators of platelet function often are vasoactive agents. For example, NO is a vasodilator and thromboxane A_2 is a vasoconstrictor. In looking for possible vascular activity of MMP-2, Fernandez-Patron *et al.* compared the cleavage sites in collagen and laminin by MMP-2 with that of the vasoactive peptide big endothelin-1 (big ET-1).[117] A striking similarity led them to hypothesize that MMP-2 is involved in the regulation of vascular tone by processing big ET-1. Incubation of big ET-1 with MMP-2 resulted in the production of a novel peptide, $ET_{1\text{-}32}$, which is distinctive from mature $ET_{1\text{-}21}$ produced by the action of endothelin converting enzymes. $ET_{1\text{-}32}$, which was termed "medium ET-1", is a much more potent vasoconstrictor compared to $ET_{1\text{-}21}$.[117] In the meantime, MMP-2 was also shown to be able to degrade calcitonin-gene related peptide, a vasodilator peptide, into inactive fragments.[118] The combined actions of MMP-2 on big ET-1 and calcitonin-gene related peptide could lead to a higher vasoconstrictive state. Indeed, inhibition of MMPs in rat mesenteric arteries resulted in vasodilation.[118] These actions of MMP-2 appear to be specific as many other vasoactive peptides were not processed by MMP-2 under their experimental conditions.

MMP-2 is able to process not only vasoactive peptides but also inflammatory mediators and growth hormone regulators. Monocyte chemoattractant protein-3 was discovered to interact with MMP-2 in a yeast-two hybrid system using a truncated MMP-2 sequence as bait. The cleavage of monocyte chemoattractant protein-3 by MMP-2 resulted in its reduced ability to induce chemotaxis and the cleavage product acts as an antagonist of monocyte chemoattractant protein receptors.[119] In this manner, MMP-2 may reduce the local inflammatory response in synovial fluids. Both MMP-2 and MMP-9 can proteolytically activate latent transforming growth factor-β and tumor necrosis factor precursor[120]. Insulin-like growth factor binding protein-3 was proteolyzed by MMP and resulted in the increased action of insulin-like growth factor[121]. In the TIMP-1 knock-out mice, higher level of insulin-like growth factor binding protein and lower effect of insulin-like growth factor has been observed[122].

Recently, MMP-2 was shown by our group to contribute to acute myocardial ischemia-reperfusion injury.[123] In isolated perfused rat hearts, MMP-2 is released into the coronary effluent during aerobic perfusion. During reperfusion after ischemia, there was a dramatic enhanced release of MMP-2 into the perfusate within the first minute. The release of MMP-2 correlated negatively with the recovery of cardiac contractile function during reperfusion in hearts subjected to increasing duration of ischemia. Inhibition of MMPs (using either o-phenanthroline, doxycycline or neutralizing anti-MMP-2 antibody) protected hearts by improving the recovery of contractile function. The regulation of MMP-2 activity during myocardial ischemia-reperfusion injury and the mechanism by which MMP-2 induces injury to the heart are not clear. This is one of the major themes of my thesis.

1.2 Nitric oxide and peroxynitrite

1.2.1 Nitric oxide physiology

In 1980, Furchgott and Zawadzki were the first to discover a novel vasodilator substance released from vascular endothelium, which they named endothelium-derived relaxing factor.[124] Endothelium-derived relaxing factor was later identified to be by Moncada's group[125]. NO is a rapidly diffusible gas molecule with an available unpaired electron. It readily crosses biological membranes making it a suitable biological messenger. In the cardiovascular system, NO primarily acts as a vasodilator and inhibitor of platelet function via cyclic GMP-dependent pathways in an autocrine or paracrine manner.[126]

Synthesis of NO from its precursor L-arginine is a two-step process, with N^G-hydroxyl-L-arginine formed as an intermediate, catalyzed by a family of enzymes called the NO synthases (NOS). To date, at least three distinct isoforms of NOS are characterized and named after the cell type in which they were originally discovered. Namely, nNOS was found in neuronal cells[127,128] and eNOS was originally found in endothelial cells.[129] iNOS is not usually expressed under normal physiological conditions but is rapidly induced in nearly all cell types with immunological stimuli.[130-134] However, later studies demonstrated that eNOS is also expressed in other cells types such as cardiac myocytes[135-138] and kidney epithelial cells.[139] nNOS has also been found in epithelial cells,[140] skeletal muscle cells,[141] pituitary cells,[142] adrenal medulla[143] and the male sex organ.[144] eNOS and nNOS are Ca^{2+}- dependent enzymes, becoming activated when intracellular Ca^{2+} levels rise in order to activate calmodulin binding to NOS. iNOS, on the other hand, contains calmodulin domain and therefore is Ca^{2+}-independent.[127,130] Co-factors such as NADPH, FMN, FAD and tetrahydrobiopterin are required for the oxidation of L-arginine.[127]

In the heart, NO is synthesized by eNOS in endothelial cells and cardiac myocytes under normal conditions, with contribution from nNOS in cardiac neurons as well[136,145]. It is a vasodilator in the coronary vasculature, a negative ionotropic agent and an anti-oxidant. There is some evidence showing the presence of nNOS in sarcoplasmic reticulum[146] and mitochondria[147] in cardiac myocytes but the identity of this NOS is still controversial[148]. NO generated in these organelles is suggested to regulate sarcoplasmic reticulum Ca^{2+} transport and mitochondrial oxidative phosphorylation, respectively. During pathological conditions, enhanced production of NO occurs when iNOS is induced in a variety of cells which make up the heart.

As a messenger in the cardiovascular system, the primary target of NO is the soluble guanylate cyclase.[149] NO activates soluble guanylate cyclase by causing a conformational change, displacing catalytic iron out of the plane of porphyrin ring, which results in the increased synthesis of cGMP.[150] cGMP activates a cGMP-dependent protein kinase, which can phosphorylate many target proteins such as a vasodilator-stimulated phosphoprotein[151] and Ca^{2+} channels in smooth muscle cells, myocardial cells[152] and platelets.[153,154] Direct and cGMP-independent actions of NO such as nitrosylation of proteins[155] and activation of cyclooxygenase 2[156] have also been reported.

NO in aqueous solution is rapidly metabolized to nitrite (NO_2^-) and nitrate (NO_3^-) in the presence of oxygen.[125,157] A large part of NO released in vivo is converted to NO_3^-

by oxyhemoglobin.[158,159] However, NO also reacts with superoxide anion ($O_2^{\bullet -}$) at nearly the diffusion-limited rate of 6.7×10^9 $M^{-1}s^{-1}$ to form peroxynitrite ($ONOO^-$).[160] Actually, it is apparent that most of the detrimental effects of NO are actually mediated by peroxynitrite[161].

1.2.2 Peroxynitrite pathophysiology

The production of peroxynitrite has been shown in many pathological conditions in which the combined upregulation of NO and superoxide occur such as ischemia-reperfusion,[162-164] atherosclerosis,[161] septic shock,[165] and neurodegenerative diseases.[166,167] The peroxynitrite anion is stable at pH>8. At physiological pH, it is rapidly protonated to form peroxynitrous acid (ONOOH).[161] Peroxynitrous acid is unstable and decomposes by homolytic fission giving rise to nitrogen dioxide and hydroxyl radicals, substances which are much more hazardous than peroxynitrite.[168] The relative stability of peroxynitrite allows it to diffuse through the cell and attack cellular targets. The targets of peroxynitrite in the cell include proteins,[169] lipids,[170] carbohydrates[171] and nucleic acids.[172] The reactions of peroxynitrite with these biomolecules have been demonstrated to include lipid peroxidation,[170,173] protein modification by oxidization of sulfhydryl groups[173,174] and nitration of tyrosine residues.[175] The detrimental effects of peroxynitrite include structural damage, enzyme dysfunction, ion channel and transporter malfunction and eventually cell death.

Unlike many enzymes inactivated by peroxynitrite such as aconitase,[176-178] glyceraldehyde 3-phosphate dehydrogenase,[179] Na^+-K^+ ATPase[180] and caspase-3,[181] MMP activity is upregulated by peroxynitrite in vitro (see section 1.2.6).

1.2.3 NO, peroxynitrite and myocardial ischemia-reperfusion injury

Reintroduction of oxygen and blood flow facilitates the production of $O_2^{\bullet -}$, as well as the biosynthesis of NO at a level well above physiological levels because of the effect of shear stress to enhance NO production via eNOS. Due to the extremely fast kinetics (k=6.7×10^9 $M^{-1}s^{-1}$) favoring peroxynitrite formation, peroxynitrite is synthesized during the first minute of reperfusion. Peroxynitrite production was shown to occur in isolated perfused hearts by Wang *et al.* using spin trap technique during reperfusion following ischemia[162] and by Yasmin *et al.* by taking advantage of the reaction of peroxynitrite with L-tyrosine.[163] Reduction of peroxynitrite formation by superoxide dismutase mimetic drugs and by low concentrations of NO synthase inhibitors were protective both in isolated perfused hearts[163,182-185] or in vivo.[186] Detoxifying peroxynitrite using glutathione (GSH) also improved the recovery of cardiac mechanical function in isolated hearts.[187] Administration of a peroxynitrite donor, 1, 3-morpholinosydnonimine-HCl (SIN-1), enhanced myocardial ischemia-reperfusion injury.[182]

NO itself serves a dual role in oxidative stress such as that seen during ischemia-reperfusion injury. At a low physiological level, it is cytoprotective by lowering Ca^{2+} concentration via cGMP-dependent mechanisms and by stopping the free radical chain propagating reactions in the lipid mem and anti-leukocyte effects. When a higher concentration of both NO and $O_2^{\bullet-}$ is produced temporarily during reperfusion after ischemia, more peroxynitrite is synthesized which is cytotoxic as mentioned above. Indeed, both low levels of NOS inhibitors such as N^G-monomethyl-L-arginine (L-NMMA, 10 μM) and NO donors such as S-nitroso-N-acetylpenicillamine (SNAP, 0.2 μM) are protective whereas higher concentrations of these agents are detrimental to the heart during ischemia-reperfusion.[163,185]

1.2.4 NO as an anti-oxidant species

The outcome of the interactions between NO and $O_2^{\bullet-}$ or peroxynitrite or their lipid oxidation products is complex and depends on the relative concentrations of each species. The consensus is that a low, physiological concentration NO is protective and high levels NO is cytotoxic. This notion is supported by the following observations.

First, oxidation of low density lipoprotein by $O_2^{\bullet-}$ and peroxynitrite is important in the pathogenesis of atherosclerosis. Exogenous addition of NO was able to inhibit $O_2^{\bullet-}$-induced low density lipoprotein oxidation in both macrophages[188] and endothelial cell[189]. A NO donor also limited the toxicity induced by peroxynitrite infusion into isolated rat hearts[190]. Blocking NO production enhanced low density lipoprotein oxidation. Dietary supplement of L-arginine, the substrate of NOS, is beneficial in atherosclerotic diseases[191]. Second, in the production of lipid peroxyl radicals, NO shows a biphasic effect[170]. When the concentration of NO is less than or equal to that of $O_2^{\bullet-}$, NO stimulates the production of lipid peroxyl radicals via the formation of peroxynitrite. When the concentration of NO exceeds that of $O_2^{\bullet-}$, the production of lipid peroxyl radicals is inhibited. This is due to the reaction between NO and lipid peroxyl radicals at a near diffusion-limited rate ($k = 1.3 \times 10^9\ M^{-1}s^{-1}$)[192] to stop the lipid radical chain propagation reactions. In this manner, an excess of NO is able to detoxify peroxynitrite-mediated reactions. Finally, deficiency of endogenous NO production is seen in many pathological conditions in which oxidative stress is involved in, such as atherosclerosis,[193] essential hypertension,[194] diabetes,[195] coronary artery disease[196] and heart failure.[191,197] However, whether reduction of basal NO production in the heart leads to oxidative stress in the isolated heart is not known.

1.2.5 NO and MMPs

NO can modulate MMP activity both at transcriptional and post-translational levels. In the transcriptional regulation of MMPs, exogenous NO has been shown to decrease the mRNA level of MMP-9 in rat mesangial cells stimulated with IL-1β. In contrast, the NO synthase inhibitor L-NMMA increased the mRNA level of MMP-9.[198] Similar findings were reported by Gurjar *et al.* in vascular smooth muscle cells.[199] The inhibition of extracellular signal-regulated kinase (Erk) by NO is suggested to be part of the mechanism.[199,200]

Many conflicting reports are in the literature about the role of NO in post-translational regulation of MMP activity. NO donors inhibited the release of MMP-2 in human platelets,[114] tumor cells,[201] and cartilage.[202] Using the NO donor, SNAP (0.01-10 μM), Sawicki *et al.* demonstrated a concentration-dependent reduction in the release of MMP-2 from activated platelets.[114] In contrast, Murell *et al.* found that SNAP activated collagenase and stromelysin in articular cartilage treated with IL-1β, TNF-α and endotoxin.[203]

The divergent reports on the action of NO may be partially resolved by the finding that peroxynitrite is able to activate MMPs.[9,204] During inflammation, larger amounts of both NO and $O_2^{\bullet -}$ are produced by inflammatory cells, therefore greatly increasing the level of peroxynitrite. It is most likely that peroxynitrite activates collagenase and stromelysin in Murell *et al.*'s studies[203]. However, the role of basal NO generation in the regulation of MMPs activity/release in the heart is not known. I will investigate this in my thesis.

1.2.6 Oxidative stress and MMPs

Oxidant species including peroxynitrite, $O_2^{\bullet -}$ and H_2O_2 are involved in the regulation of MMP activity[22,205-208]. This is believed to be due to their ability to break the cysteine-zinc bond. Latent MMP-1, MMP-8 and MMP-9 can be activated by exogenous peroxynitrite in cultured fibroblasts[207,209] and in normal skin and tumor tissue in mouse[210]. Another possibility is S-glutathiolation of MMPs via disulfide S-oxide formation by reaction with peroxynitrite in the presence of glutathione[204]. The activation of proMMPs by peroxynitrite occurs without loss of the propeptide. These suggest that the term "proMMPs" is insufficient to describe the potential proteolytic ability of native MMPs. Therefore, it would be better to define MMPs by their molecular weight forms than to label them as "pro" or "active" forms. Interestingly, peroxynitrite can also inactivate natural inhibitors of MMPs including TIMP-1.[211] Therefore, tissue MMP activity is apparently enhanced after exposure to exogenous peroxynitrite or enhanced endogenous biosynthesis of peroxynitrite.

Both oxidant species and MMPs are upregulated side-by-side in many pathologies such as atherosclerosis, diabetes, heart failure and myocardial infarction. Antioxidants not only prevented the increase in MMP activity but also stabilized atherosclerotic plaques.[212] More recently, antioxidant therapy aiming at reducing MMPs activity is also suggested as a possible component of the treatment regimen of chronic heart failure.[213]

In many pathological conditions, increased production of pro-oxidant species overwhelms the cellular anti-oxidant defenses. This situation is called oxidative stress. Oxidants such as reactive oxygen species, reactive nitrogen species and lipid peroxides may cause detrimental effects to living cells via activation of MMPs. This is another major theme of my thesis.

1.3 Myocardial ischemia-reperfusion (stunning) injury

1.3.1 History and definition

In 1975, Heydrickx *et al.* first described a puzzling observation in conscious dogs.[214] Occlusion of the left anterior descending artery resulted in severe depression of function. After release of a 5 min occlusion and reperfusion, the ST segment elevation disappeared completely in less than 1 min, however, the regional function remained significantly depressed for 2 hr. A 15 min occlusion caused derangement of function in the ischemic region for a more prolonged duration of 6 hr.[214] This was the first description of prolonged recovery of cardiac contractile function after full restoration of coronary flow following a brief period of ischemia. Braunwald and Kloner later coined the term "myocardial stunning injury" to describe this phenomenon.[215] However, this finding initially did not receive much interest from researchers because reperfusion of the ischemic myocardium was not considered a likely scenario seen in the clinical practice at that time. Until almost a decade later, with the advance of thrombolytic techniques[216] and invasive cardiac interventions,[215] myocardial ischemia and reperfusion was considered to be a critical situation encountered by physicians routinely. Much attention was then given to the pathophysiology of the myocardial stunning injury.[217,218] The hope resides in better understanding the mechanism and finding a better way to prevent the injury.

Myocardial stunning injury is defined as postischemic mechanical dysfunction persisting after normal or near-normal restoration of coronary flow despite the absence of irreversible damage.[218] Two important aspects in this definition are: 1) full restoration of coronary flow and 2) viable, non-infarcted myocardium with full recovery of function after a short or long period of reperfusion.

1.3.2 Reactive oxygen species and myocardial stunning injury

Many theories have been proposed to explain myocardial stunning injury. Oxidative stress, calcium overload and metabolic derangement hypotheses remain the most plausible and promising candidates.[218] None of them can exclusively explain all the phenomena of myocardial ischemia-reperfusion injury. The widely held belief is that these theories overlap with each other and each can explain one part of stunning injury.

During ischemia, the cessation of oxygen supply enhances the production of reactive oxygen reactive species such as $O_2^{\bullet -}$, H_2O_2 and $OH^{\bullet}$[219,220]. Mitochondrial uncoupling may be the main source of $O_2^{\bullet -}$ during ischemia. Reintroduction of oxygen to the myocardium after ischemia leads to a burst-like production of both reactive oxygen and nitrogen species (discussed in section 1.2.3). These reactive molecules can attack proteins, lipids and deoxyribonucleic acid which are critical to cellular functions.[217,221-223] Indeed, administration of a cell permeable superoxide dismutase mimetic alone or together with catalase significantly improved the recovery of cardiac mechanical function in hearts subjected to ischemia and reperfusion.[224-226] Scavengers of $OH^{\bullet}$ such as dimethylthiourea[227] and mercaptopropionyl glycine[228] or the ion chelator desferrioxamine[229,230] were also found to be protective in the stunned myocardium. Evidence of the production of oxygen-derived radicals in stunned myocardium was demonstrated by spin trap studies.[219] Taken together, all these evidences support the notion that oxidative stress is part of the pathophysiology of myocardial stunning injury.

1.3.3 Contractile proteins and myocardial stunning injury

The cardiac myocyte contractile apparatus consists of the thin filament, the thick filament and the Z-disc anchoring the adjacent sarcomeres. The components of the thin filament are actin, tropomyosin and troponin. Troponin is made up of three subunits, i.e. troponin I (TnI), troponin T (TnT) and troponin C (TnC). The thick filament is made up of the "golf-club"-like myosin molecules. In the Z-disc, proteins such as α-actinin, desmin and vimentin are proposed to maintain the myofibrils in order. Titan is the largest known molecule with a molecular weight of 3600-kDa. It extends from the thick filament to the Z-disc, acting like a molecular spring to keep the thick filament centered.[231] The active sliding of the thick filaments along the thin filaments, regulated by troponin, results in contraction and relaxation of cardiac myocytes. In these processes, the interaction between the regulatory subunit TnI and the Ca^{2+}-binding subunit TnC plays a critical role.[232] Figure 1.3 illustrates the cardiac contractile proteins.

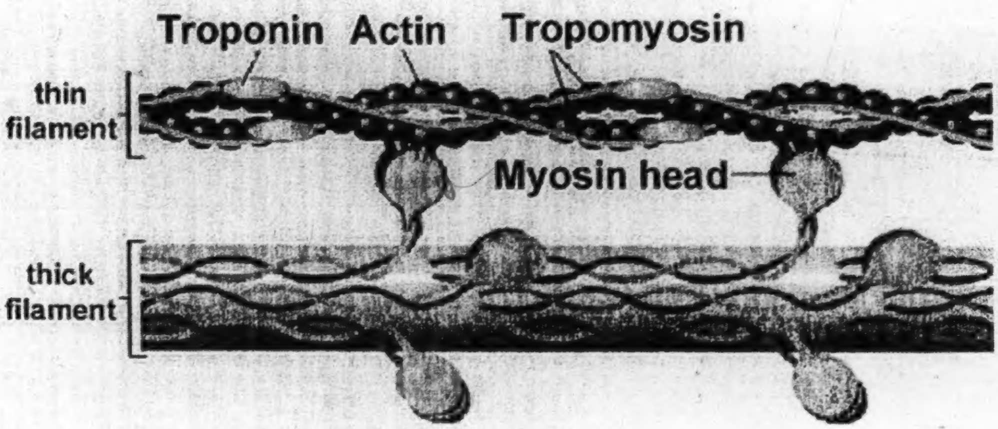

Figure 1.3 Cardiac contractile apparatus.

In clinical practice, the elevation of TnI and TnT in the plasma has been widely accepted as a specific marker for myocardial infarction. In severe ischemic insult (infarction), TnI and TnT have been suggested to be susceptible to degradation.[233] Calpain, a calcium-activated protease, may contribute to the degradation of TnI in severe ischemia (≥ 60 min duration).[234] Two isoforms of calpain have been identified with different Ca^{2+} concentrations required for their activation, namely μ-calpain (μM Ca^{2+} required)[235] and m-calpain (mM Ca^{2+} required)[236]. m-calpain has been shown to be able to degrade TnI in vitro.[237] Calpain activation in the isolated rat heart has only been shown to occur with a long period of global ischemia (60 min) followed by 30 min reperfusion.[238] TnI degradation fragments have also been identified in serum from patients who suffered an acute myocardial infarction [239,240]. What should be pointed out is that the immunoassay used clinically for the detection of TnI does not differentiate between native TnI or its fragments[241].

Proteolysis of contractile proteins has also been proposed to be part of the pathological mechanism of stunning injury. The proteins in the list include TnI,[242-244] TnT,[244] α-actinin,[243,245] myosin heavy chain and myosin light chain[243] desmin[245,246] and spectrin,[245] with TnI as the best studied molecule to date. In 1997, Gao *et al.* first proposed that TnI is degraded in a model of stunning with brief ischemia (20 min) followed by 30 min reperfusion.[242] In the following years, many independent investigators have confirmed their observation. The evidence for the involvement of TnI degradation in stunning injury is accumulating. Van Eyk *et al.* identified one of TnI fragments as $TnI_{1\text{-}193}$ in isolated rat hearts subjected to a long duration of ischemia (60 min).[244] Murphy *et al.*overexpressed this fragment in transgenic mice, resulting in a phenotype which show depressed cardiac contractility resembling features of stunning injury.[247] In myocardial biopsy sample from patients undergoing cardiopulmonary bypass surgery, a scenario mimicking acute ischemia-reperfusion injury, the proteolysis of TnI was also demonstrated.[247,248]

However, TnI proteolysis has not been observed in some species such as dog[249] and pig.[250] Multiple reasons could explain this apparent discrepancy. First, different stunning mechanisms occur in different species. A quite different pathogenesis may underlie stunning injury in pigs.[250,251] Second, the consistency of techniques such as immunoblot and SDS-PAGE is not guaranteed. Different antibodies might have varied affinity to TnI or to its degradation fragments. Third, the experimental conditions are so varied that a direct comparison between studies is difficult. The experimental settings used include isolated myocytes, skinned trabecular fibers, isolated heart perfusion, open-chest experiments, etc. The duration of regional or global ischemia and reperfusion varied too. Clearly, more carefully designed experiments and new techniques such as genetic manipulation are required to conclude whether TnI proteolysis is a key element of myocardial stunning injury.

The enzyme(s) responsible for the proteolysis of TnI is not clear. Many questions have been raised against the hypothesis that calpain is responsible for the proteolysis of TnI during myocardial ischemia-reperfusion injury. First, the enzyme responsible for TnI_{1-193} formation in the study by McDonough *et al.* is not known.[244] There is no evidence indicating that calpain is able to cleave TnI at the N^{193}-I^{194} bond. Second, the activation and/or upregulation of calpain during a brief period of ischemia associated with myocardial stunning injury was not observed[252]. It is possible that the regulation of calpain activity is tightly controlled through calpastatin, its endogenous inhibitor[252,253]. An interesting observation by Rouet-Benzineb *et al.* demonstrating that myosin heavy chain is degraded by MMP-9 in dilated cardiomyopathy led me to ask the question whether MMPs may be responsible for the degradation of cardiac contractile proteins[74]. In summary, it is reasonable to speculate there are other enzymes, including MMPs, which may be responsible for the degradation of contractile proteins during myocardial ischemia-reperfusion injury. This will be explored in my thesis.

1.3.4 Isolated heart perfusion as a myocardial ischemia-reperfusion model

The term "stunning" covers many scenarios such as global, no-flow ischemia, single or repetitive regional ischemia and exercise-induced ischemia.[217] Slightly different pathogeneses probably underlie these situations. One of the experimental models widely used is the isolated perfused heart subjected to a brief period of global, no-flow ischemia. According to the method described by Langendorff, hearts are perfused at a constant pressure or, alternatively, at a constant flow by means of an infusion pump, via an aortic cannula with Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2. Hearts are subjected to a brief (typically 20 min) global ischemia by clamping the in-flow line, during which time provisions are made to keep hearts at 37°C. reperfusion is introduced by restoring the flow of perfusate aerated with 95% O_2 and 5% CO_2. The noncirculating feature allows for the convenient collection of coronary effluent samples for biochemical measurements. This is a widely used stunning model without introducing infarction or necrosis.[218] One limitation of this model as a model of stunning is the full recovery of cardiac mechanical function is not seen due to the short total perfusion time allowable when the heart is perfused with crystalloid solutions (normally less than 2 hr).

1.4 Hypotheses and objectives

The understanding of the novel regulation and biological effects of MMPs in oxidatively stressed hearts is a central objective which links the various studies in this thesis. The primary experimental model used is the isolated rat heart perfused according to the Langendorff technique. *My overall hypotheses are oxidants such as peroxynitrite activate MMPs in the heart, that there is an imbalance between TIMPs and MMPs during myocardial ischemia-reperfusion and that MMP-2 contributes to myocardial ischemia-reperfusion by cleavage of TnI.*

1. Peroxynitrite-induced myocardial injury is mediated through matrix metalloproteinase-2.

It is known that the release of MMPs from the heart is acutely increased during reperfusion following ischemia, and that blockade of MMP activity improves the recovery of cardiac mechanical function.[123] Yasmin *et al.* previously showed that peroxynitrite, the reaction product of NO and $O_2^{\bullet-}$, is released during acute reperfusion following ischemia and contributes to the impairment in myocardial mechanical function and coronary vascular tone.[162,163,182] Peroxynitrite was shown to be a potent activator of the zymogen form of MMPs to an active enzyme.[9,204] Thus I hypothesize that infusion of peroxynitrite into the heart will activate MMPs and contribute to cardiac contractile dysfunction.

1.1 Infusion of peroxynitrite will cause depression in cardiac mechanical function in a concentration-dependent manner.

1.2 Infusion of peroxynitrite will increase the release of MMP activity.

1.3 Detoxifying peroxynitrite or inhibition of MMP activity will prevent the depression in cardiac mechanical function.

2. Inhibition of nitric oxide synthases in the heart enhances matrix metalloproteinase-2 release via oxidative stress

The basal production of NO in the coronary vasculature functions as an anti-oxidant defense. Removing basal NO production by infusion of a NO synthase inhibitor will introduce a state of oxidative stress in the heart. As oxidant stress is able to activate MMPs, I hypothesize that *infusion of L-NAME will increase MMP activity in hearts via oxidative stress*.

3. The imbalance between TIMPs and MMPs during acute myocardial ischemia-reperfusion injury

In many long-term cardiac pathologies, an imbalance between TIMPs and MMPs has been shown, resulting in a proteolytic state within tissue. Activation of MMP-2 contributes to acute myocardial ischemia-reperfusion. The alteration in myocardial TIMPs during this process is not known. I hypothesize that:

3.1 There is an imbalance between TIMPs and MMPs during reperfusion after ischemia in the myocardium.

3.2 Net tissue gelatinolytic activity increases in hearts subjected to ischemia-reperfusion.

4. Tnl is a target of MMP-2 in acute myocardial ischemia-reperfusion injury

The proteolytic degradation of TnI is known to participate in the pathogenesis of myocardial ischemia-reperfusion injury.[123] The enzyme responsible for this proteolysis is not known. The molecular target of MMP-2 in the heart is also not clear. I will investigate the following hypotheses:

4.1 *TnI is susceptible to proteolytic attack by MMP-2 in vitro.*

4.2 *TnI content is reduced in hearts subjected to ischemia-reperfusion.*

4.3 *Inhibition of MMPs prevents the loss of TnI and improves the recovery of cardiac mechanical function.*

4.4 *There is an intracellular association between TnI and MMP-2.*

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CHAPTER II

MATERIALS AND METHODS

This chapter contains materials and methods common to all of the studies presented in this thesis. Those materials and methods specific to one study are given in the relevant chapter. These investigations conform to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (revised 1993).

2.1 Materials

2.1.1 Agents

Conditioned culture medium from a human fibrosarcoma cell line HT-1080 (American Type Culture, Rockville, MD), which contains large amounts of MMP-2, was used as a standard in gelatin zymography. HT-1080 cells were maintained at 37°C in a humidified chamber containing 5% CO_2 in air in Eagle's minimum essential medium with 10% fetal calf serum at a concentration of 5×10^5 cells/ml. The cells were washed 3 times with serum-free media and incubated at 37°C for 24 hr with phorbol ester 12-o-tetradecanoyl-phorbol-13-acetate (0.1 μM). The cell-conditioned medium was separated from cells by centrifugation (1500 *g* for 10 min at room temperature), and the supernatant was aliquoted and stored at -80°C. All other agents, unless indicated, with purity higher than 99% were obtained from Sigma Chemical Co. (St Louise, MO).

2.1.2 Antibodies

A polyclonal anti-peptide antibody was generated in rabbits using synthetic peptide corresponding to a fragment (N-G-K-E-Y-N-S-[ABA]-T) of the gelatin-binding domain of human MMP-2 (a gift from Dr Mieczyslaw Wozniak, Department of Clinical Chemistry, Medical University, Wroclaw, Poland). Antibody was purified by affinity chromatography using protein A-sepharose.

2.2 Heart preparation and perfusion

Male Sprague-Dawley rats (250-300 g) were used for the experiments. Hearts were rapidly excised from pentobarbital anesthetized rats and briefly rinsed by immersion in ice-cold Krebs-Henseleit solution. They were perfused via the aorta either at constant flow (10 ml/min) by means of a peristaltic pump (Buchler Instruments Inc, Fort Lee, NJ) or at constant hydrostatic pressure (60 mmHg) with Krebs-Henseleit solution at 37 °C. The composition of the buffer was (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (3.0), $NaHCO_3$ (25), glucose (11), EDTA (0.5), pH 7.4. It was continuously gassed with 95% O_2/5% CO_2.

Spontaneously beating hearts were used in all experiments. A water-filled latex balloon connected to a pressure transducer (Model 60-3002, Harvard Apparatus, Southnatick, MA) was inserted into the left ventricle through an incision in the left atrium and through the mitral valve and the volume was adjusted to achieve an end diastolic pressure of 8-12 mmHg. A pressure transducer was placed in the infusion line close to the heart to monitor coronary perfusion pressure. Coronary perfusion pressure, heart rate and left ventricular pressure were monitored on an IBM compatible computer using an MP100 system (BIOPAC System Inc., Santa Barbara, CA). Coronary flow was measured by a flow probe placed in the inflow line close to the aortic cannulae (Model T206, Transonic System Inc., Ithaca, NY). Left ventricular developed pressure was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace. The rate-pressure product (RPP) was calculated as the product of heart rate and left ventricular developed pressure. A water-jacketed glass chamber was positioned around the heart to maintain its temperature at 37°C. Solutions of various reagents were infused into the heart via a side-port proximal to the aortic cannulae at a flow rate of 0.1 ml/min by a Gilson mini pump (Minipuls 3, Villiers Le Bel, France).

The intervals between thoracotomy and attachment of the heart to the perfusion system and between thoracotomy and beginning of stabilization period were less than 1 and 5 min, respectively. Hearts with following parameters were excluded: heart rate lower than 200 beats/min, left ventricular developed pressure lower than 60 mmHg or coronary perfusion pressure either lower than 30 or higher than 70 mmHg. Using this perfusion protocol, hearts maintained a steady state of coronary flow, heart rate and left ventricular developed pressure for at least 80 min after stabilization.

2.3 Perfusion protocol

2.3.1 Peroxynitrite infusion

Hearts were perfused at constant flow of 10 ml/min. Following 20 min of perfusion (stabilization period), the thromboxane mimetic U46619 (1-100 nM) was infused at a concentration sufficient to increase the coronary perfusion pressure to 100-150 mmHg from a baseline of 40 mmHg. The infusion of U46619 was maintained throughout the duration of the perfusion. After 15 min of U46619 infusion, when the increase in coronary perfusion pressure reached a plateau in all hearts, either decomposed peroxynitrite or peroxynitrite (30 or 80 μM) were infused into the hearts for 15 min followed by a 15 min washout period. Hearts were perfused for a total duration of 65 min and their mechanical function was recorded. Six ml of coronary effluent was collected immediately before and 1, 2, 5, 10, and 15 min after beginning the infusion of peroxynitrite and at the end of the washout period (samples were collected over 36 seconds period immediately before the designated time). Coronary effluent samples were immediately placed on ice and processed on the same day. At the end of the perfusion, hearts were freeze-clamped with tongs cooled to the temperature of liquid nitrogen and stored at -80°C.

In separate groups of hearts, either the MMP inhibitor, PD-166793 (2 μM) or glutathione (GSH, 300 μM) were infused into the heart at the same time with U46619. S-nitroso-acetylpenicillamine (SNAP, 3 μM) was infused into a separate group of hearts for 15 min in a similar manner as peroxynitrite. Coronary effluent samples were collected at the same time points as in the peroxynitrite group.

2.3.2 Infusion of NOS inhibitor

Hearts were perfused at a constant flow of 10 ml/min. Following 20 min of perfusion (stabilization period), the thromboxane mimetic U46619 (9.1 ± 2.2 nM) was infused at a concentration sufficient to increase the coronary perfusion pressure to ~60 mmHg from a baseline of 40 mmHg. The infusion of U46619 was maintained throughout the duration of the perfusion. After 15 min of U46619 infusion, when the increase in coronary perfusion pressure reached a plateau in all hearts, either N^G-nitro-L-arginine methyl ester (L-NAME, 10 or 100 μM each) or vehicle were infused into the hearts for 15 min followed by a 15 min washout period. Hearts were perfused for a total duration of 65 min and their mechanical function was recorded. Six ml of coronary effluent was collected immediately before, and 15 min after beginning the infusion of L-NAME and at the end of the washout period. Coronary effluent samples were immediately placed on ice and processed on the same day. At the end of the perfusion, hearts were freeze-clamped with tongs cooled to the temperature of liquid nitrogen and stored at -80°C. SNAP (1 μM) was co-infused with 100 μM L-NAME for the same duration into a separate group of hearts.

A higher concentration of U46619 (37.8 ± 14.5 nM) was infused as a control for the vasoconstrictor effect of L-NAME into a separate group of hearts. Coronary effluent samples were collected as above.

2.3.3 Ischemia-reperfusion protocol

Hearts were perfused at constant hydrostatic pressure of 60 mmHg. After 25 min of stabilization during aerobic perfusion, hearts were subjected to either 15, 20 or 25 min of global, no-flow ischemia (at 37 °C) followed by 30 min aerobic reperfusion. Control hearts were perfused aerobically for 75 min. Samples of coronary effluent (6 or 12 ml) were collected immediately before ischemia and after 1, 5, 10, and 30 min of reperfusion (samples were collected over a 30-120 sec period). Hearts were freeze-clamped in liquid nitrogen at the end of perfusion and stored at –80°C. Protein extraction and concentration measurements were performed as described below.

2.4 Concentration of proteins in the coronary effluent

The coronary effluent samples were concentrated in Centricon-30 (Centricon-10 was used for detection of TIMPs) concentrating vessels (Amicon Inc, Beverly, MA). These units have a membrane with a molecular weight cutoff of 30 and 10-kDa, respectively. Thus solutes with a molecular weight of ~ 30-kDa (Centricon-30) or ~10-kDa (Centricon-10) and greater remain in the concentrator chamber. The vessels were spun at 5000*g* at 4°C for 4-9 hr. After concentration, the final volume of samples was measured gravimetrically. Protein concentrations in the concentrate were determined by bicinchoninic acid assay using bovine serum albumin as a standard. The protein concentration in the original coronary effluent before concentration was calculated and expressed as μg/ml.

2.5 Preparation of heart extracts

Frozen hearts were crushed using a mortar and pestle at liquid nitrogen temperature and then homogenized by sonication in ice-cold 50 mM Tris-HCl (pH 7.4) containing 3.1 mM sucrose, 1 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, and 0.1% Triton X-100. The homogenate was centrifuged at 10,000 *g* at 4°C for 10 min and the supernatant was collected and stored at -80°C.

2.6 Gelatin zymography

Gelatin zymography was performed as previously described[1]. Briefly, concentrated coronary effluent samples were mixed with non-reducing sample loading buffer, and applied to 8% polyacrylamide gels copolymerized with 2 mg/ml gelatin. One to two μg of effluent concentrate or 40 μg of myocardial tissue extract were loaded in each lane. After electrophoresis, gels were rinsed in 2.5% Triton X-100 (3× 20 min) to remove SDS. Then the gels were washed twice in incubation buffer for 20 min each at room temperature. The composition of the incubation buffer was (in mM): Tris-HCl (50), $CaCl_2$ (5), NaCl (150) and 0.05% NaN_3. The gels were then kept in incubation buffer at 37°C overnight. After incubation, gels were stained in staining solution (2% Coomassie Brilliant blue G, 25% methanol, 10% acetic acid) for 2 hr and then destained twice for 30 min each in destaining solution (2% methanol/4% acetic acid). Zymograms were scanned using an HP 6100 scanner (Hewlett-Packard), and the band intensities were analyzed using Sigmagel software (version 1.0, Jandel Scientific). MMP activities were expressed as activity per μg protein in either the coronary effluent or the myocardial extract.

2.7 Conventional electron microscopy

At the end of perfusion, hearts were perfused manually using a 60 ml syringe with 4% paraformaldehyde-0.1% glutaraldehyde in phosphate buffer saline over 10 min and were then removed from the perfusion apparatus and cut into pieces of 1×1×3 mm. The segments were then fixed again in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH

7.4, at room temperature for 2.5 hr). All the following steps, unless indicated otherwise, were performed at room temperature. Segments were fixed again with 1% osmium tetroxide in phosphate buffered saline for 2 hr. After washing in distilled water and dehydration in a graded series of ethanol solutions (50%, 70%, 90% and 100%, 10 min each) before two final dehydration steps in absolute ethanol. Staining was carried out in 70% ethanol containing 1% uranyl acetate for 30 min. After segments were treated with absolute propylene oxide (3 × 10 min), they were embedded in propylene oxide containing Araldite CY212 (Marivac Ltd., Halifax, Canada). Ultrathin sections (0.1 μm) were cut using an Ultracut E (Reichert-Jung, Wien, Austria). The ultrathin sections were stained in 4% uranyl acetate for 15 min and then 0.2% lead citrate for 5 min. The sections were washed in running distilled water using a device described previously (2 × 30 min)[2]. The sections were examined with a Hitachi H-7000 transmission electron microscope (Tokoyo, Japan) at 75 kV.

2.8 Electron microscopy with immunogold labeling

Five rat hearts (20′ I/R) were perfusion fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffered saline and cut as discussed above. After dehydration in increasing concentrations of ethanol (50% to absolute), specimens were embedded in Unicryl (British BioCell International, Cardiff, United Kingdom) and polymerized with ultraviolet radiation at 4 °C overnight. All the following procedures were performed at room temperature. Nickel grids with ultrathin sections were floated on 1% glycine/1% bovine serum albumin in phosphate buffered saline (pH 7.2). After transferring the grids to a Falcon 30340 microtest plate and incubating them with either rabbit polyclonal anti-MMP-2, or rabbit anti-TIMP-4 or isotype control IgG (4 μg/ml each), they were incubated for 4 hr. Sections were then rinsed with 1% bovine serum albumin in phosphate buffered saline and placed on drops of anti-rabbit IgG gold conjugate (1:20, Sigma, St. Louis, MO) in 1% bovine serum albumin/0.5% Tween in phosphate buffered saline for 2 hr. Grids were stained with 2% uranyl acetate for 15 min and then with 0.2% of lead citrate for 5 min. The grids were washed and examined as described above.

2.9 Isolation of thin myofilaments

Thin myofilament preparations from aerobic perfused and 20′ ischemic-reperfused rat hearts were isolated as described by Spiess *et al.*[3] Briefly, three hearts in each group were pooled, minced and sonicated in 2 volumes of salt buffer [100 mM KCl, 3 mM $MgCl_2$, 10 mM imidazole, 5 mM dithiothreitol, pH 7.3, and a blend of protease inhibitors (Complete Mini, 1 tablet per 10 ml, Roche Diagnotics, Mannheim, Germany)] on ice. All of the following centrifugation and resuspension procedures were performed at 4°C. After centrifuging at 10,000*g* for 5 min, the supernatant was discarded and the pellet was slowly resuspended with a rotator in 2 volumes salt buffer (w:v) containing Triton X-100 (0.6%) for 1 hr at 4°C. The suspension was centrifuged at 10,000*g* for 5 min and the supernatant was discarded. The resuspension and centrifugation step was repeated 4 times. Then the pellet was resuspended in salt buffer only and centrifuged as above another 4 times. The resulting pellet was suspended in 4 volumes of buffer 1 (100 mM KCl, 3 mM $MgCl_2$, 3 mM EDTA, 5 mM imidazole, 5 mM dithiothreitol, pH 7.5) and spun at 10,000*g* for 5 min. The supernatant was discarded and the pellet was resuspended in 0.5 volume (w:v) of buffer 1 with 5 mM ATP and rapidly homogenized with a sonicator on ice. The suspension was centrifuged at 5000*g* for 10 min and the pellet was discarded. The supernatant was spun at 30,000*g* for 30 min and the pellet was saved. The resulting supernatant was then spun at 50,000*g* for 30 min and the pellet was kept. The supernatant was centrifuged again at 80,000*g* for 180 min and the pellet was saved. The pellets were dissolved separately in 100 µl non-reducing sample loading buffer (without bromphenol blue). Protein concentrations of the supernatant were measured by the bicinchoninic acid assay using bovine serum albumin as a standard (Sigma Chemical Co. St Louis, MO). Each of the resuspended fractions were separately analyzed by SDS polyacrylamide gel electrophoresis followed by Coomassie blue staining in order to confirm the purity of the preparations. The 80,000*g* pellet contained the highest purity thin myofilament fraction and was used for further analysis. Gelatin zymography was performed on the same preparation in order to determine MMP activity.

2.10 Statistical analysis

Data are expressed as mean ± standard error of the mean. One and Two-way, single or repeated measures, ANOVA with Student-Neuman-Keuls post hoc test or Student's *t*-test were used for statistical analysis as appropriate. A value of p less than 0.05 was considered statistically significant.

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CHAPTER III

PEROXYNITRITE-INDUCED MYOCARDIAL INJURY IS MEDIATED THROUGH MATRIX METALLOPROTEINASE-2

A version of this chapter has been published. Wenjie Wang, Grzegorz Sawicki & Richard Schulz. Peroxynitrite-induced myocardial injury is mediated through matrix metalloproteinase-2. Cardiovasc Res 2002; 53: 165-174.

3.1 Introduction

NO and superoxide ($O_2^{\bullet -}$) react at a diffusion-limited rate to form peroxynitrite[1]. As a potent oxidant species, peroxynitrite is recognized to play a key role in many cardiac pathologies such as ischemia-reperfusion injury[2-5] and pro-inflammatory cytokine-induced myocardial dysfunction[6]. Indeed, antioxidants such as glutathione detoxify peroxynitrite and protect hearts from ischemia-reperfusion injury[7]. Many biological molecules such as proteins[8], lipids[9], carbohydrates[10] and nucleic acids[11] can react with and are modified by peroxynitrite and therefore are considered to be its targets. Many enzymes are inactivated and lose their function such as aconitase[12]and Na^+-K^+-ATPase[13] upon exposure to peroxynitrite. In contrast, the latent forms of matrix metalloproteinases (MMPs) are known to be activated by oxidant species including peroxynitrite[14,15].

MMPs are a family of zinc-containing endopeptidases with at least 20 members and are best recognized for their ability to degrade the extracellular matrix[16]. While all MMPs are synthesized as pro-enzymes (pro-MMPs), most of them are secreted from the cell as pro-MMPs and are activated pericellularly.[17] Breakage of the cystenyl sulphydryl bond between a cysteine residue of the pro-peptide and the Zn^{2+} catalytic centre is necessary for the activation of the zymogen form.[18] This can be achieved by either proteolytic cleavage of part of the pro-peptide, resulting in an active enzyme with 8-10 kDa lower mass, by conformational changes induced by denaturing agents such as sodium dodecyl sulfate[18] or by oxidant stress molecules like peroxynitrite[14]. For example, peroxynitrite was shown to be a potent activator of pro-MMP-8 without losing the pro-peptide[14]. The activities of MMPs are also regulated by endogenous inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs)[16].

Apart from roles of MMPs in long-term remodeling processes such as embryonic development, wound healing and cancer invasion[19], there is increasing evidence that some MMPs like MMP-2 can also rapidly regulate diverse cellular functions independent of their effects on the extracellular matrix[20-24]. This includes effects of MMP-2 on platelet aggregation[20], the control of vascular contractile tone through the proteolytic effects of MMP-2 on big endothelin-1[21] and calcitonin gene related peptide[22] and attenuation of inflammatory signals by cleavage of monocyte chemoattractant protein-3[23]. We recently demonstrated that MMP-2 activity is acutely enhanced in isolated perfused rat hearts during reperfusion following ischemia and contributes in part to myocardial stunning[24]. The mechanism of this enhancement remains obscure. Because the time course of the acute enhancement in peroxynitrite and MMPs in the heart in the first minute of reperfusion following ischemia are similar[3,24], we hypothesized that peroxynitrite activates MMPs in the myocardium and that the mechanical dysfunction of heart subjected to the infusion of exogenous peroxynitrite is mediated in part by MMPs.

3.2 Materials and methods

3.2.1 Materials

The supernatant from phorbol ester activated human fibroblast HT1080 cells (American Type Culture Collection, Rockville, MD), which contains large amounts of pro-MMP-2, MMP-2, pro-MMP-9 and MMP-9 was used as a standard. Phorbol ester induces MMP-9 expression in these cells. The MMP inhibitor PD-166793 was a kind gift from Parke-Davis (Ann Arbor, MI). Peroxynitrite and decomposed peroxynitrite were freshly prepared as previously described before each experiment[3]. Briefly, an ice-cold solution of $NaNO_2$ (2 M) and a mixed solution of nitric acid (11.1M) and H_2O_2 (8.2 M) in two separate syringes were simultaneously discharged into a rapidly stirring solution of excess ice-cold NaOH (4.2 M). Decomposed peroxynitrite was prepared by discharging the $NaNO_2$ solution and nitric acid/H_2O_2 mixture into an empty beaker on ice. After allowing 5 min to ensure complete decomposition of peroxynitrite, the same volume of NaOH solution as used for the preparation of peroxynitrite was added. Decomposed peroxynitrite contains NO_2^- and NO_3^- at the equivalent pH and NaOH concentration as peroxynitrite. The solutions were filtered through $Mn(IV)O_2$ to remove excess H_2O_2. The concentration of peroxynitrite and verification of decomposed peroxynitrite was determined by UV spectroscopy ($\lambda_{max} = 302$ nm; $\varepsilon = 1670\ M^{-1} \times cm^{-1}$). Stock solutions were prepared by dilution in double distilled water and were kept in a light-shielded container during the infusion.

3.2.2 Heart preparation and perfusion

Hearts were perfused at constant flow of 10 ml/min. See sections 2.2 and 2.3.1 in Chapter II for details.

3.2.3 Concentration of proteins in the coronary effluent

See section 2.4 in Chapter II.

3.2.4 Preparation of heart extracts

See section 2.5 in Chapter II.

3.2.5 Measurement of MMPs by gelatin zymography

See section 2.6 in Chapter II.

3.2.6 Inhibition of MMPs by PD-166793 in vitro

To investigate the inhibitory profile of PD-166793 on the gelatinolytic activities of MMPs in coronary effluent samples, PD-166793 (0.2-20 μM) was added to the incubation buffer during the overnight incubation period.

3.3 Results

3.3.1 Effect of peroxynitrite on coronary vascular tone and cardiac mechanical function

Infusion of 30 μM peroxynitrite caused vasodilatation evidenced as a decline in coronary perfusion pressure which peaked at 10 min, was maintained for the duration of the 15 min infusion and was reversed upon washout (Figure 3.1a). Infusion of 80 μM peroxynitrite caused a more rapid vasodilatation which peaked at 5 min, and then rapidly converted to vasoconstriction which was maintained even after washout of peroxynitrite. Only slight vasoconstriction, which was not statistically significant, was seen in hearts infused with decomposed peroxynitrite (Figure 3.1a). The 15 min infusion of 80 μM, but not 30 μM peroxynitrite, also caused a slowly developing and significant depression of cardiac mechanical function. This was due mainly to a decrease in LVDP, accompanied by a slight but insignificant decrease in heart rate (Table 3.1). In contrast, hearts treated with decomposed peroxynitrite showed no change in mechanical function (Figure 3.1b). Fifteen minutes after stopping the infusion of 80 μM peroxynitrite, cardiac mechanical function remained significantly depressed compared with hearts treated with decomposed peroxynitrite (Figure 3.1b).

3.3.2 Effect of peroxynitrite on coronary effluent and myocardial MMP-2 levels

In accordance with our previous study [24], all coronary effluents at baseline from aerobically perfused hearts showed a strong band of 72-kDa gelatinolytic activity (Figure 3.2a), whereas only some effluents showed a much weaker band of 64-kDa activity (data not shown). While infusion of 30 μM peroxynitrite did not significantly increase 72-kDa gelatinolytic activity, infusion of 80 μM peroxynitrite caused a rapid increase in this activity in the coronary effluent. This increase did not occur in the effluent from hearts infused with decomposed peroxynitrite (Figure 3.2a and b). The 72-kDa activity peaked at 10 min of peroxynitrite infusion and subsequently declined. After washout, the release of gelatinolytic activity from the heart returned to baseline levels. Moreover, infusion of peroxynitrite caused an increase in total protein release from the hearts (Figure 3.2c), however, this became statistically significant only at 15 min infusion, after the peak increase in 72-kDa gelatinolytic activity (Figure 3.2b). There was no difference over time in the protein concentration in the effluent from both decomposed peroxynitrite- and 30 μM peroxynitrite-treated hearts (Figure 3.2c).

There was no discernable change in tissue 72-kDa gelatinolytic activity levels between 80 μM peroxynitrite or decomposed peroxynitrite -treated hearts either at 10 min infusion or at the end of washout period (130 ± 28 vs 139 ± 31, 122 ± 12 vs 118 ± 32 arbitrary units/μg protein, respectively, $p > 0.05$, n=6).

3.3.3 Effect of glutathione on MMP-2 release and myocardial function of peroxynitrite - treated hearts

The antioxidant glutathione (300 μM) blocked the increase of 72-kDa activity in the coronary effluent (Figure 3.3a), and prevented the decline in cardiac mechanical function caused by infusion of 80 μM peroxynitrite (Figure 3.3b). In accordance with our previous results[7], peroxynitrite caused only prolonged vasodilatation in the presence of glutathione (data not shown).

3.3.4 Effect of MMP inhibitor on MMP-2 release and myocardial function of peroxynitrite-treated hearts

PD-166793, when added to the zymography incubation buffer, concentration-dependently inhibited the 72-kDa activity of a 35 min perfusion coronary effluent sample from an aerobically perfused heart (Figure 3.4a). PD-166793 did not alter the coronary vascular response to peroxynitrite, neither did it have an effect on the concentration of peroxynitrite during its in vitro incubation with peroxynitrite for 15 min at room temperature (data not shown). The hearts co-infused with both peroxynitrite and PD-166793 still showed increased release of 72-kDa gelatinolytic activity (Figure 3.4b). This is expected because of the ready dissociation of the complex between MMP-2 and PD-166793 under electrophoretic conditions (data not shown). PD-166793 (2 μM) abolished the depression of cardiac mechanical function induced by 80 μM peroxynitrite (Figure 3.4c).

3.3.5 Effect of SNAP on coronary vascular tone and MMP-2 release

Peroxynitrite reacts with endogenous thiols such as glutathione forming nitrosothiol intermediates which then release nitric oxide[25,26]. To verify whether the enhancement of 72-kDa activity in the coronary effluent by peroxynitrite was due to vasodilatation or due to NO formation, we used the nitrosothiol NO donor S-nitroso-acetylpenicillamine (SNAP, 3 μM, n=5) to induce vasodilatation to a similar level as seen in peroxynitrite -treated hearts and did not find any change in MMP activity in the coronary effluent. Infusion of SNAP caused a rapid and reversible vasodilatation without having any effect on mechanical function (Figure 3.5a). However, it caused no change in the release of 72-kDa gelatinolytic activity (Figure 3.5b).

3.4 Discussion

We have shown here that infusion of authentic peroxynitrite caused a rapid and significant increase in 72-kDa gelatinolytic activity into the coronary effluent which preceded the decline in myocardial contractile function. Detoxification of peroxynitrite with glutathione prevented not only the increase in 72-kDa activity but also the myocardial dysfunction. Inhibition of MMP-2 with PD-166793 protected hearts from peroxynitrite -induced myocardial depression. Our findings suggest a novel role of MMP-2 in mediating peroxynitrite -induced injury to the heart.

In spite of its short half-life, the heart was exposed to a constant concentration of peroxynitrite by its continuous infusion into the perfusion buffer via a side-port immediately proximal to the aortic cannula. In a similar model as used here, Vila *et al.*[27] gave bolus injections of 100 μM peroxynitrite over 30 sec into isolated rat hearts and found that it caused vasodilatation. This vasodilatory response to peroxynitrite showed rapid desensitization upon repeated injection. Similarly, we found that continuously infusion of 80 μM peroxynitrite caused an initial vasodilatation which then converted into vasoconstriction. This effect of peroxynitrite was also concentration-dependent as 30 μM peroxynitrite infusion showed only prolonged vasodilation (Figure 3.1a). No discernible change of coronary perfusion pressure was observed in hearts treated with decomposed peroxynitrite. It is plausible that a nitrosothiol NO donor is produced by the reaction of endogenous tissue thiols with peroxynitrite which then initiates the vasorelaxation[27].

In accordance with our previous report[24], the main gelatinolytic activity in coronary effluent from the aerobically perfused heart is 72-kDa, corresponding to pro-MMP-2. We did not see any 92-kDa gelatinolytic activity (MMP-9) either in coronary effluent or heart homogenates even with longer (72 hr) incubation of the zymograms. This reflects the notion that MMP-9 is primarily found in neutrophils[28] and macrophages[29]. A rapid and significant increase in 72-kDa activity was observed in coronary effluent from hearts exposed to 80 μM peroxynitrite. Moreover, this enhancement was an early event induced by peroxynitrite and unrelated to a general increase in the level of myocardial proteins in the coronary effluent, which peaked several minutes later (Figure 3.2). Neither was it related to the formation of a nitrosothiol intermediate as the release of MMP-2 activity was not stimulated by S-nitroso-acetylpenicillamine, a nitrosothiol which nonetheless caused a similar decrease in coronary perfusion pressure as peroxynitrite. Furthermore, exogenously supplied glutathione prevented the increase in coronary effluent gelatinolytic activity and protected the heart from peroxynitrite -induced myocardial dysfunction at the same concentration at which it protected hearts from endogenous peroxynitrite -mediated injury caused by ischemia and reperfusion[7]. Nor is this effect of peroxynitrite due to coronary vasoconstriction, because we have found that there was no relation between coronary vasoconstriction and MMP-2 release (see Chapter IV). Thus the pro-oxidant property of peroxynitrite appears to be responsible for the enhancement in 72-kDa activity.

That we did not observe any concomitant loss in tissue 72-kDa activity following peroxynitrite infusion may have several reasons: a) ischemia-reperfusion is a stronger insult to the heart and it resulted in a great release of 72-kDa activity[24]; b) ischemia-reperfusion results in oxidative stress due to endogenous peroxynitrite likely to be formed directly within the cardiomyocytes, in contrast to the intravascular application used here, and c) the release of 72-kDa activity appears to be a subtle yet early response to an intravascular oxidative stress.

Peroxynitrite is able to enhance MMP activity by several means. As it is recognized for its ability to oxidize thiols[30], peroxynitrite might cause oxidative modification of the cysteine residue in the pro-peptide domain. In the presence of glutathione, the reaction of peroxynitrite with MMPs results in their activation by S-glutathiolation, causing the formation of a S-oxide[31]. Perturbation of the cysteine-zinc bond, the so called cysteine switch, is suggested to underlie the activation mechanism of pro-MMPs[18]. The zymogen form of purified human neutrophil collagenase (pro-MMP-8) was activated by authentic peroxynitrite in a concentration range similar to what was used here [14] without causing a change in its molecular weight. It was also speculated that nitration of tyrosine residues in the hinge region between the pro-peptide and the catalytic domain could also assist in the unfolding of pro-MMPs [15]. Although inactivation of TIMPs by peroxynitrite may result in the upregulation of gelatinolytic activity[32], it could not explain our findings because TIMPs should be dissociated from MMP-2 under the conditions of the zymography assay. Whether in vivo inactivation of TIMPs by peroxynitrite contributes to the enhancement in MMP activity needs further investigation, as peroxynitrite was shown to inactivate TIMP-1 in vitro[32].

Not surprisingly, Owens *et al.*[33] observed loss of MMP-2 activity with high concentrations of peroxynitrite ($\geq 250\ \mu M$). It is compatible with the notion, as shown here, that a low level of oxidant stress may activate certain enzymes such as MMPs, whereas higher levels of oxidant stress by peroxynitrite could incur further protein modifications (i.e. thiol oxidation, nitrosylation, nitrotyrosine formation) which result in loss of enzyme activity[12,34]. Very high concentrations of peroxynitrite ($\geq 500\ \mu M$) are able to directly induce protein fragmentation in cell-free systems[35, 32, 33].

The mechanism of myocardial injury induced by MMPs remains unclear. Apart from the extracellular matrix, little is known about other targets of MMPs[16]. Recently, myosin heavy chain was found to be susceptible to degradation by MMP-2 in vitro and evidence for degradation fragments were found in hearts from patients with dilated cardiomyopathy[36]. We have evidence showing that a contractile protein regulatory element, troponin I, is susceptible to proteolytic cleavage by MMP-2 and that MMP-2 is co-localized with troponin I in ischemic-reperfused hearts (see Chapter VI)[37]. Moreover, inhibitors of MMPs prevented the degradation of troponin I in ischemic-reperfused hearts as well as improved the recovery of mechanical function. Whether contractile protein elements such as myosin or troponin I are degraded during peroxynitrite -induced myocardial injury requires further study.

Peroxynitrite is implicated in the pathogenesis of several cardiovascular pathologies including ischemia-reperfusion injury[3-5], pro-inflammatory cytokine-induced myocardial contractile failure[6], doxorubicin-induced cardiac toxicity[38], autoimmune-induced myocarditis[39], acute allograft rejection[40], cardiomyocyte apoptosis[41] and atherosclerosis[42]. Interestingly, MMPs have also been implicated in many cardiovascular diseases, particularly, ischemia-reperfusion injury (both acute,[24] and chronic[43]), heart failure[44] and the vascular derangement in atherosclerosis[45]. We speculate that enhancement of peroxynitrite generation precedes the activation of MMPs, and therefore some of the detrimental effects of peroxynitrite are mediated by activation of these proteases. Indeed, antioxidant therapy using N-acetyl-cysteine prevented gelatinase activation in atherosclerotic aorta of hypercholesterolemic rabbits[46]. Specific drug targeting of peroxynitrite and/or MMPs could prove useful therapeutic regimens in the treatment of these diseases.

In summary, we have demonstrated that peroxynitrite rapidly increased the release of MMP-2 activity in the coronary effluent. Inhibition of MMP activity or detoxifying peroxynitrite with glutathione protected the heart from peroxynitrite -induced myocardial injury. This provides a new insight into the pathological mechanism of myocardial dysfunction where peroxynitrite biosynthesis is enhanced.

Table 3.1 Effects of 15 min infusion of either decomposed (dec.) or peroxynitrite (30 and 80 μM) on heart rate (HR) and left ventricular developed pressure (LVDP).

		Baseline	2 min	5 min	10 min	15 min	Washout
dec. peroxynitrite n=6	HR (beats/min)	330 ± 12	336 ± 11	331 ± 9	302 ± 38	305 ± 37	334 ± 9
	LVDP (mmHg)	54 ± 11	49 ± 14	49 ± 11	46 ± 10	50 ± 10	51 ± 10
peroxynitrite 30 μM n=3	HR (beats/min)	331 ± 13	331 ± 18	328 ± 11	326 ± 19	322 ± 17	333 ± 15
	LVDP (mmHg)	53 ± 10	53 ± 12	61 ± 15	60 ± 9	60 ± 5	56 ± 7
peroxynitrite 80 μM n=6	HR (beats/min)	311 ± 6	321 ± 12	304 ± 7	268 ± 27	264 ± 29	259 ± 36
	LVDP (mmHg)	57 ± 4	55 ± 2	61 ± 5	49 ± 4	29 ± 7*	29 ± 10*

*$p < 0.05$ vs baseline by one way ANOVA with Neuman-Keuls post hoc test

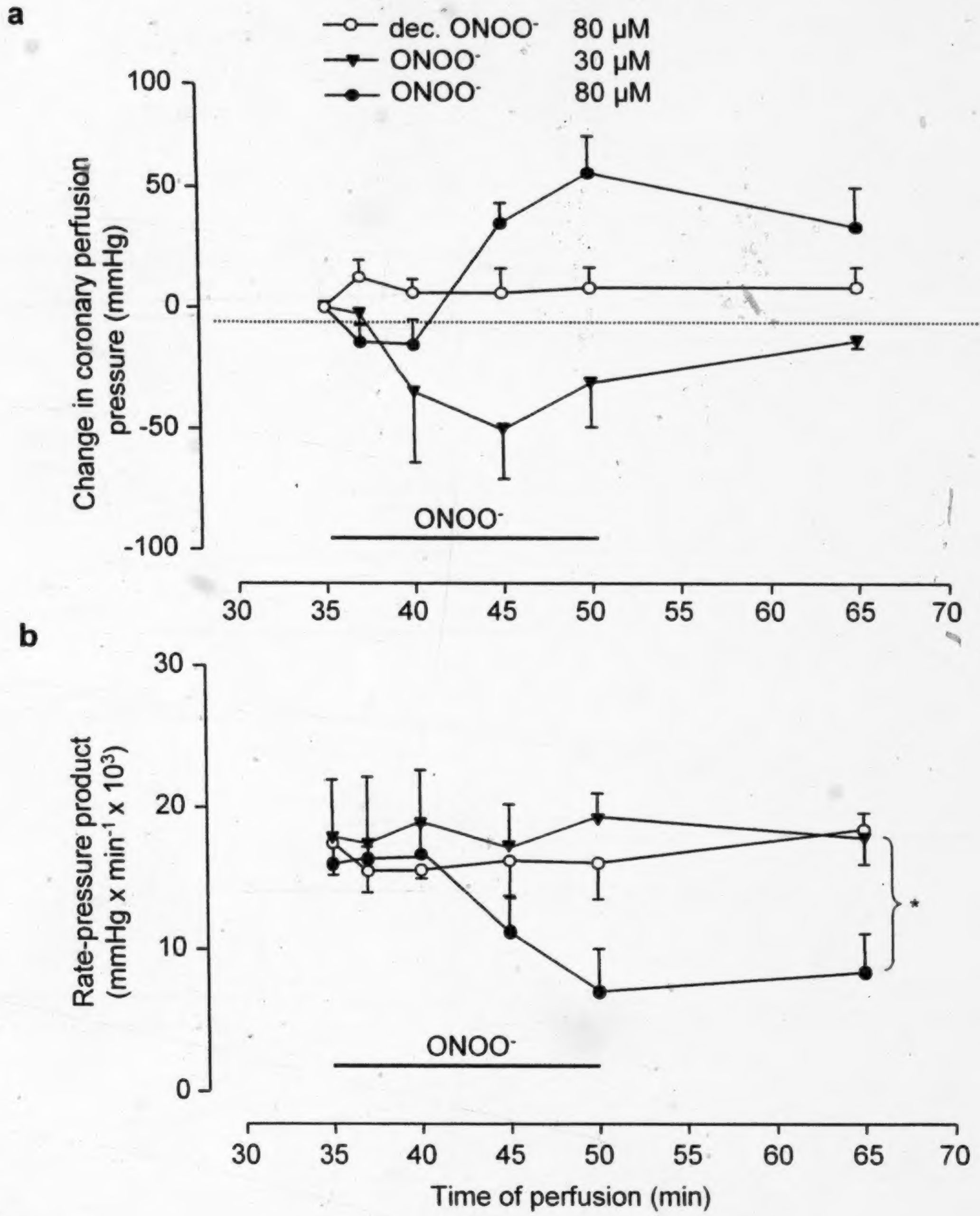

Figure 3.1 Effects of peroxynitrite ($ONOO^-$, 30 and 80 µM) or decomposed $ONOO^-$ (80 µM) on coronary perfusion pressure (a) and mechanical function measured as the rate-pressure product (heart rate × left ventricular developed pressure) (b) in isolated perfused rat hearts. Bar denotes infusion of $ONOO^-$ or decomposed (dec.) $ONOO^-$. * $p < 0.05$ vs. decomposed $ONOO^-$ by two-way repeated measures ANOVA, n=3-6 per group

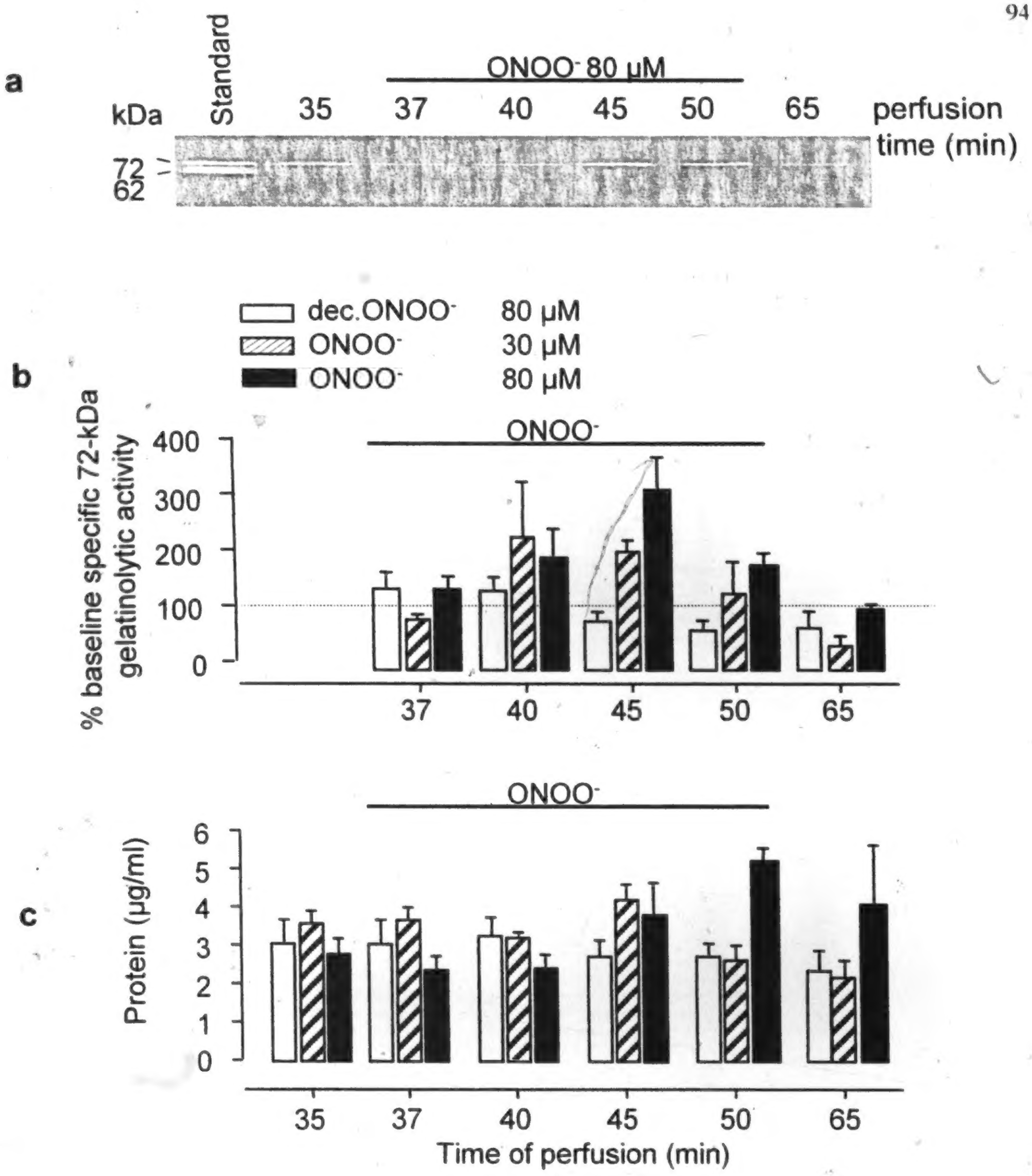

Figure 3.2 Time-dependent effects of ONOO$^-$ (30 and 80 μM) on the release of gelatinolytic activity as well as total protein into the coronary effluent. (a) Representative zymogram of coronary effluent samples from ONOO$^-$-treated hearts. Standard indicates HT1080 cell-conditioned medium. (b) Summary data quantifying 72-kDa activity from hearts infused either ONOO$^-$ or decomposed (dec.) ONOO$^-$. (c) Total protein concentration in the coronary effluent. Bar denotes infusion of ONOO$^-$ or dec. ONOO$^-$. For both panels b and c, the differences between 80 μM ONOO$^-$ and decomposed ONOO$^-$ are $p < 0.05$ by two-way repeated measures ANOVA, n= 6 in each group.

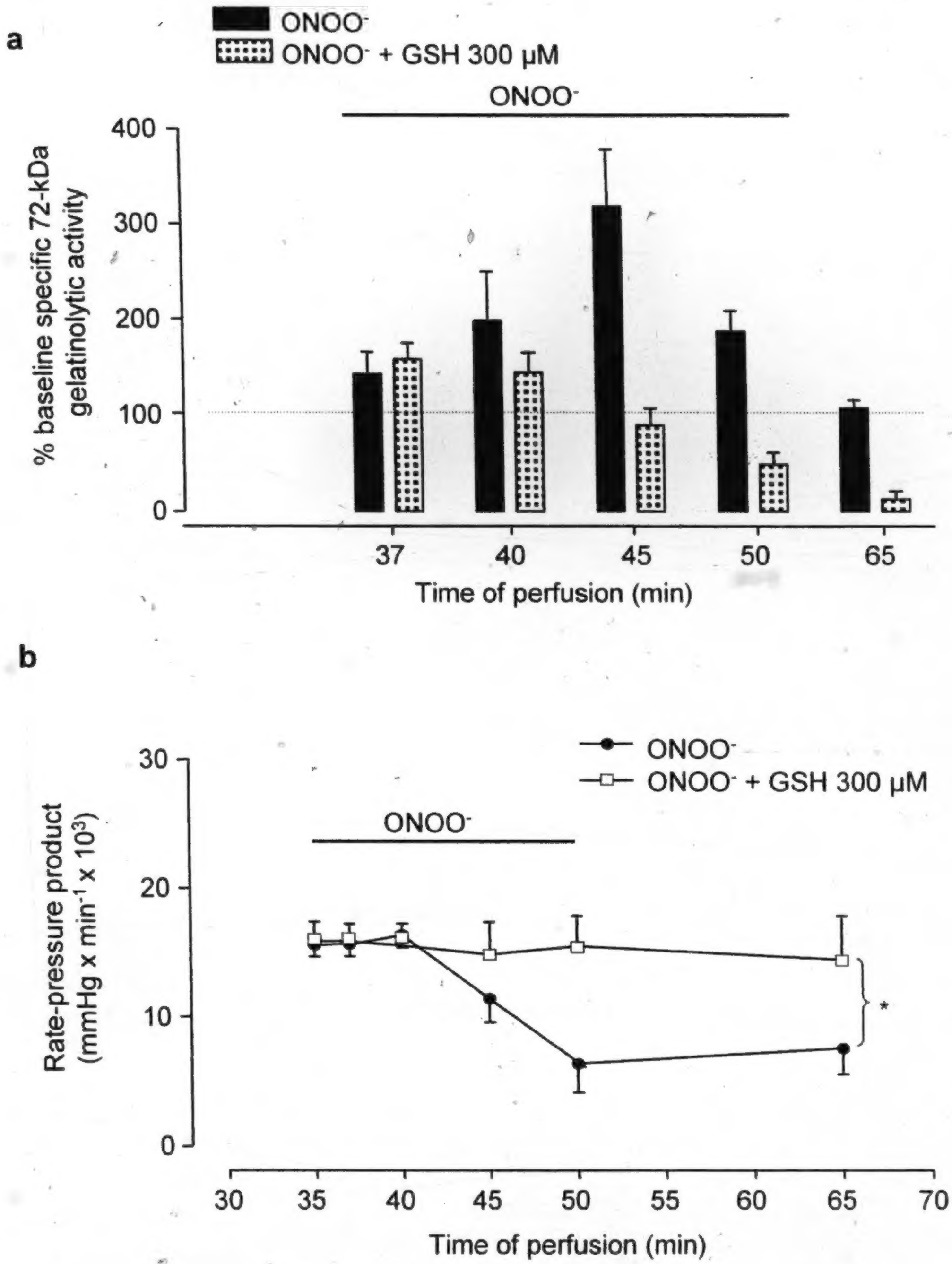

Figure 3.3 Effects of 300 μM glutathione (GSH) infusion on changes of 72-kDa gelatinolytic activity in coronary effluent from hearts subjected to 80 μM $ONOO^-$ (a) and cardiac mechanical function (b).

Differences between $ONOO^-$ and $ONOO^-$ + GSH for both panels are $p < 0.05$ by two-way repeated measures ANOVA, n=5-6.

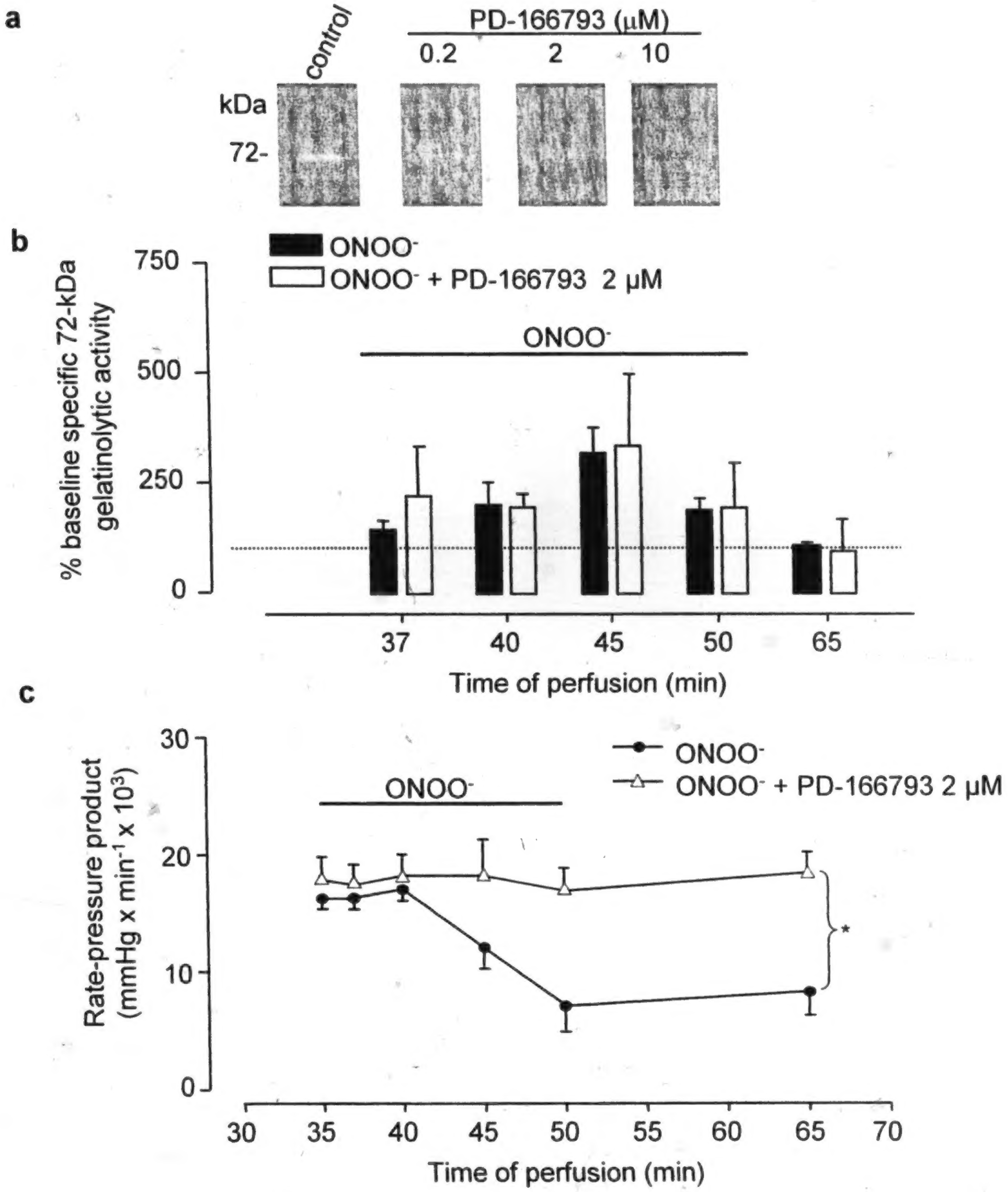

Figure 3.4 (a) Inhibitory profile of PD-1666793 on MMP activities in a coronary effluent sample from aerobically perfused rat hearts taken at 35 min perfusion. (b) Effect of 2 μM PD-166793 on MMP-2 release stimulated by 80 μM ONOO$^-$ infusion into the coronary effluent. (c) Effect of 2 μM PD-166793 on mechanical function measured as rate-pressure product from hearts subjected to 80 μM ONOO$^-$.

*$p<0.05$ vs. ONOO$^-$ by two-way repeated measures ANOVA, n=6 in each group

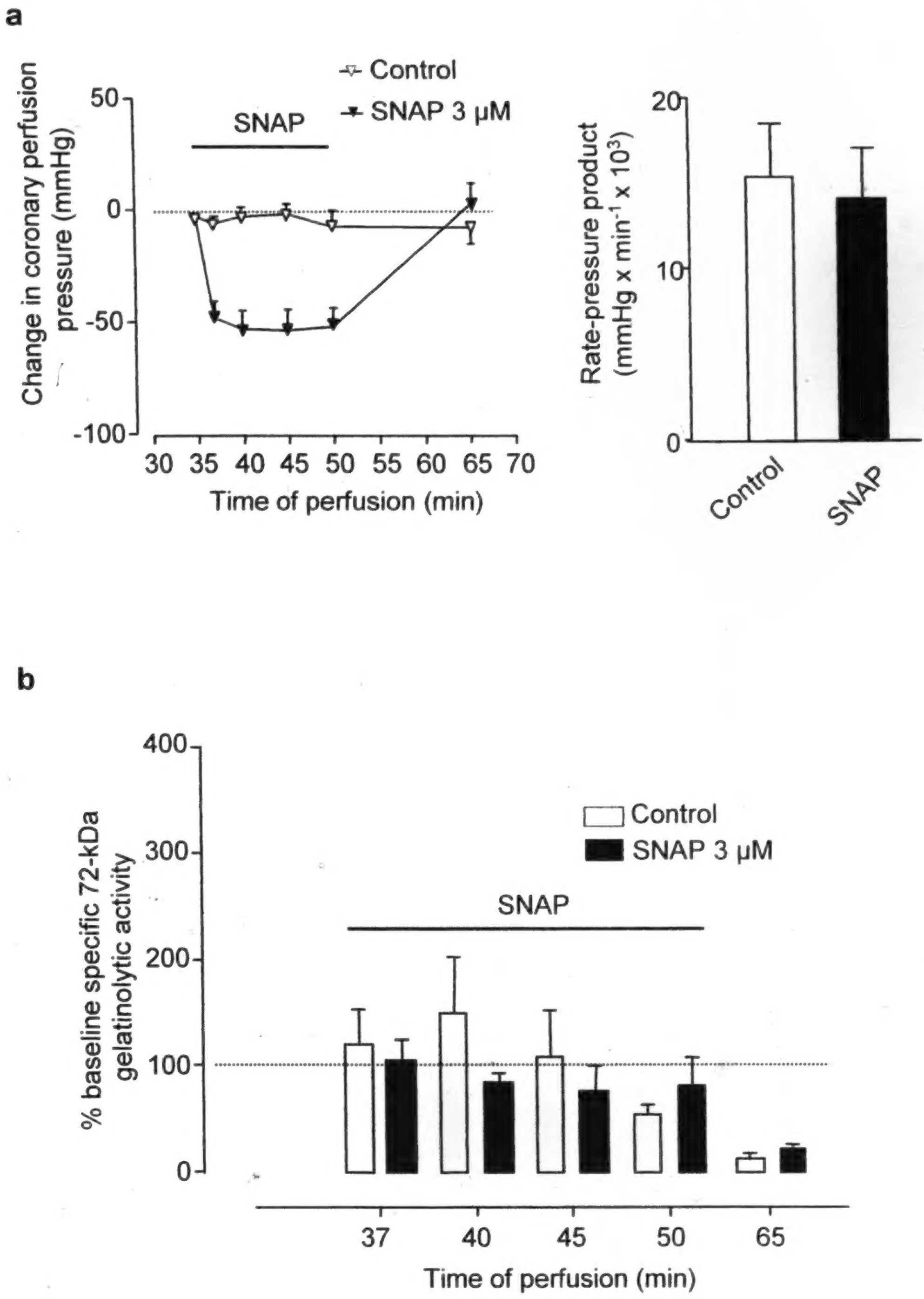

Figure 3.5 (a) Effects of 3 μM SNAP or vehicle on coronary perfusion pressure (left panel) and mechanical function (right panel). (b) Effect of SNAP on MMP-2 release into coronary effluent. n=5 each.

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CHAPTER IV

INHIBITION OF NITRIC OXIDE SYNTHASES IN THE HEART ENHANCES MATRIX METALLOPROTEINASE-2 RELEASE VIA OXIDATIVE STRESS

A version of this chapter has been submitted for publication. Wenjie Wang, Jolanta Sawicka, Rick Schulz. Inhibition of nitric oxide synthases in the heart enhances matrix metalloproteinase-2 release via oxidative stress. British Journal of Pharmacology.

4.1 Introduction

NO is an important biological messenger with a variety of roles in the cardiovascular system. Its biological functions in the heart have been identified including vasodilation, anti-platelet and neutrophil actions, modulation of cardiac contractile function and oxygen consumption and others (see review[1]). Moreover, endogenous NO is an important antioxidant defense in the body. Although NO combines with superoxide at a diffusion-limited rate to form the cytotoxic molecule peroxynitrite,[2] NO itself has been shown to counteract peroxynitrite-mediated cytotoxicity.[3,4] Second, NO is able to prevent ischemia-reperfusion damage in heart.[5,6] One mechanism by which this occurs is by inhibiting the propagation of free radical chain reactions in the lipid membrane.[7-9] Deficiency of endogenous NO biosynthesis or its enhanced degradation may lead to greater oxidative stress seen in many pathological conditions such as atherosclerosis,[10] essential hypertension,[11] diabetes,[12] coronary artery disease[13] and heart failure.[14,15]

MMPs are zinc-containing endopeptidases which are comprised of at least 25 members and are best recognized for their ability to degrade components of the extracellular matrix.[16] Activation of their zymogen forms is a very important step in the regulation of their activity. Breakage of the cystenyl sulfhydryl bond between a cysteine residue of the pro-peptide and the Zn^{2+} catalytic centre is necessary for the activation of the zymogen form.[17] This can be achieved by either cleavage of the pro-peptide by other proteases including MMPs, resulting in an active enzyme with 8-10 kDa lower mass, or by conformational changes induced by denaturing agents such as sodium dodecyl sulfate.[18] More recently oxidant stress molecules like peroxynitrite have been shown to activate MMPs without cleavage of the pro-peptide. This mechanism includes a novel S-glutathiolation of the MMP, resulting in the disruption of a zinc-cysteine bond.[19]

We have found that there is a basal release of MMP-2 into the coronary effluent in aerobically perfused isolated rat hearts. The release of MMP-2 is rapidly enhanced in the first minute of reperfusion following ischemia (a state of enhanced oxidative stress) and contributes to the immediate impairment in myocardial contractile function.[20] Infusion of peroxynitrite into isolated rat hearts caused a rapid increase in MMP-2 release into coronary effluent which impaired contractile function, the latter, which could be blocked by the antioxidant glutathione or by inhibiting MMP activity.[21] Although exogenous NO has been shown to inhibit the release of MMPs from platelets,[22] tumor cells[23] and cartilage,[24] the interaction of NO and MMPs in the intact coronary circulation remain elusive. We hypothesized that basal NO generation in the coronary vasculature may regulate MMP-2 release via its antioxidant properties and tested this using a NOS inhibitor strategy in isolated perfused rat hearts.

4.2 Materials and methods

4.2.1 Heart preparation and perfusion

Hearts were perfused at constant low of 10 ml/min. See sections 2.2 in Chapter II for details.

4.2.2 Infusion of NO synthase inhibitors

See sections 2.3.2 in Chapter II.

4.2.3 Concentration of proteins in the coronary effluent

See section 2.4 in Chapter II.

4.2.4 Measurement of MMPs by gelatin zymography

See section 2.6 in Chapter II.

4.2.5 Lipid hydroperoxide assay

As a measure of oxidative stress, lipid hydroperoxide content of heart tissue was determined using a commercial kit based on a colorimetric assay, as previously described.[25] After deproteinization and extraction of the samples with methanol and chloroform, the absorbance was determined using a quartz 96-well plate at 500 nm. The level of lipid hydroperoxide was expressed as pmol/mg protein.

4.3 Results

4.3.1 Effect of L-NAME on coronary vascular tone and MMP-2 release into coronary effluent

Infusion of N^G-nitro-L-arginine methyl ester (L-NAME, 10 or 100 μM) for 15 min increased coronary vascular tone in a concentration-dependent manner as demonstrated by an increase in the coronary perfusion pressure by 19.4 ± 8.3 and 47.5 ± 10.7 mmHg respectively (Figure 4.1a, Table 4.1). Infusion of L-NAME at these concentrations did not have any effect on cardiac mechanical function (Table 4.1). In accordance with our previous reports,[20,21] the only MMP activity readily detectable by gelatin zymography in the perfusate is 72-kDa MMP-2. A 62-kDa MMP-2 is seen in some perfusate samples when a longer incubation time during zymography is used. Therefore in this study, we focused on the release of 72-kDa MMP-2 from the heart. The baseline MMP-2 activity, defined as 100%, was determined at 35 min perfusion, the time point immediately before the 15 min infusion of L-NAME. During the perfusion of control hearts, the release of MMP-2 gradually declines by 50 min to 36.2 ± 7.4 % of the baseline level. Infusion of L-NAME concentration-dependently increased the release of MMP-2 (to 46.5 ± 8.7% and 83.7 ± 18.8 % of baseline in 10 and 100 μM L-NAME groups, respectively, Figure 4.1b).

4.3.2 Effect of co-infusion of SNAP with L-NAME on coronary vascular tone and MMP-2 release into coronary effluent

Co-infusion of 1 μM SNAP with L-NAME (100 μM) for 15 min not only abolished the increase in coronary perfusion pressure but also prevented the enhanced release of MMP-2 (Figure 4.2).

4.3.3 Effect of U46619 on coronary vascular tone and MMP-2 release

Infusion of a higher concentration of U46619 (37.8 ± 14.5 nM) to induce a similar increase in coronary perfusion pressure as that of 100 μM L-NAME did not alter the level of MMP-2 in the coronary effluent (Figure 4.3).

4.3.4 Effect of treatments on lipid hydroperoxide level in heart tissue

Infusion of L-NAME concentration-dependently increased the level of lipid hydroperoxides in the myocardium (Figure 4.4). The infusion of 100 μM L-NAME caused a significant increase in the level of lipid hydroperoxides. The effect of 100 μM L-NAME was abolished by co-infusion of 1 μM SNAP.

4.4 Discussion

Here we report that the basal generation of NO regulates the release of MMPs in the coronary vasculature. In many pathological conditions, either reduced basal NO biosynthesis or its enhanced degradation have been extensively documented. We show here that a reduction in endogenous NO leads to a higher state of oxidative stress in the coronary vasculature which in turn activates MMPs, particularly MMP-2. This study provides the first evidence of an important interaction between NO and metalloproteinases in the heart.

NO generated by the coronary vasculature serves as one of the antioxidant defenses in the heart. Evidence for this statement comes from the following observations. NO donors were able to reduce ischemia-reperfusion damage, an insult associated with enhanced oxidative stress. Infusion of authentic NO decreased myocardial infarct size in cats after reperfusion following occlusion of the left anterior descending artery[5] whereas SNAP at a subvasodilatory concentration prevented myocardial stunning injury in isolated rat hearts.[4] After occluding the middle cerebral artery, infusion of the substrate for NO biosynthesis, L-arginine, reduced infarct size in spontaneously hypertensive rats.[6] Wink et al demonstrated that NO directly protected both Chinese hamster lung fibroblasts and mesencephalic cells from cytotoxicity induced by the oxidative stress of hydrogen peroxide.[26]

Whether removal of basal generation of NO causes an oxidative stress in the intact heart is not known. We infused a NOS inhibitor L-NAME into isolated rat hearts to reduce the basal generation of NO in this study. As previously described, infusion of L-NAME concentration-dependently increased coronary vascular tone.[27] Infusion of L-NAME also increased the level of lipid hydroperoxides in the myocardium, an index for enhanced oxidative stress. Co-infusion of SNAP with L-NAME not only restored coronary vascular tone but also reduced the lipid hydroperoxide levels in the heart. The lack of effect of raising coronary perfusion pressure with U46619 to an equivalent level to that of L-NAME shows that the response is not related simply to enhanced perfusion pressure. Taken together, blocking NO generation caused an oxidative stress to the heart.

Activation of MMPs can be achieved either by proteolytic cleavage of an inhibitory pro-peptide domain or by conformational changes of the protein induced by chemical agents to expose the zinc atom in its catalytic core. Oxidants like peroxynitrite were able to induce conformational change and therefore activate the zymogen form of MMPs without a loss of the propeptide domain[19,28]. Indeed, infusion of peroxynitrite into isolated rat hearts increased the release of 72-kDa activity in the coronary effluent[21] prior to the reduction in contractile function of the heart. In the present study, infusion of L-NAME not only increased lipid hydroperoxide levels but also MMP-2 release, suggesting that oxidants might induce the release of MMP-2. The prevention of both the increase in lipid hydroperoxides and the enhanced MMP-2 release by replenishing the loss of endogenous NO with SNAP further supports this notion. Rajagopalan *et al.* demonstrated the activation of MMP-2 by hydrogen peroxide in vitro[29] and Siwik *et al.* showed increased MMP-2 activity in cardiac fibroblasts exposed to the agent.[30]

Endothelial dysfunction has been suggested to be involved in many diseases such as atherosclerosis, essential hypertension,[11] diabetes,[12] coronary artery disease[13] and heart failure (see review[15]). In 1986, Ludmer *et al.* first documented a paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries.[10] Following this study, many observations have been reported to support endothelial dysfunction. Both decreased NO synthesis and increased production of oxidants with the capability to inactivate NO have been suggested in the etiology of endothelial dysfunction. The final result is an increase in oxidative stress. Interestingly, enhanced MMP activity has also been demonstrated in atherosclerosis.[31] Vascular MMP activity is also believed to play an important role in the rupture of plaques. Antioxidants not only prevented the increase in MMP activity but also stabilized atherosclerotic plaques.[32] More recently, antioxidant therapy to reduce MMP activity is also suggested as a possible treatment of chronic heart failure.[33] In summary, blocking the generation of basal NO induces oxidative stress and increases MMP activity in the heart. Our data suggest a novel interaction between NO and MMPs in the heart.

Table 4.1 Effects of treatments on heart rate, left ventricular developed pressure (LVDP) and coronary perfusion pressure (CPP) in isolated perfused rat hearts.

	Heart rate (beats/min)	LVDP (mmHg)	CPP (mmHg)
Control (n = 8)	295 ± 14	76.8 ± 9.1	58.5 ± 2.8
L-NAME 100 μM (n = 8)	276 ± 13	80.9 ± 6.7	110.5 ± 11.4*
L-NAME 100 μM + SNAP 1 μM (n = 6)	283 ± 13	73.5 ± 6.1	56.5 ± 5.7†

Data were recorded at 50 min perfusion time, at the end of 15 min infusion of drugs. * $p < 0.05$ vs control, † $p < 0.05$ vs L-NAME group by one way ANOVA with Student-Neuman-Keuls post hoc test.

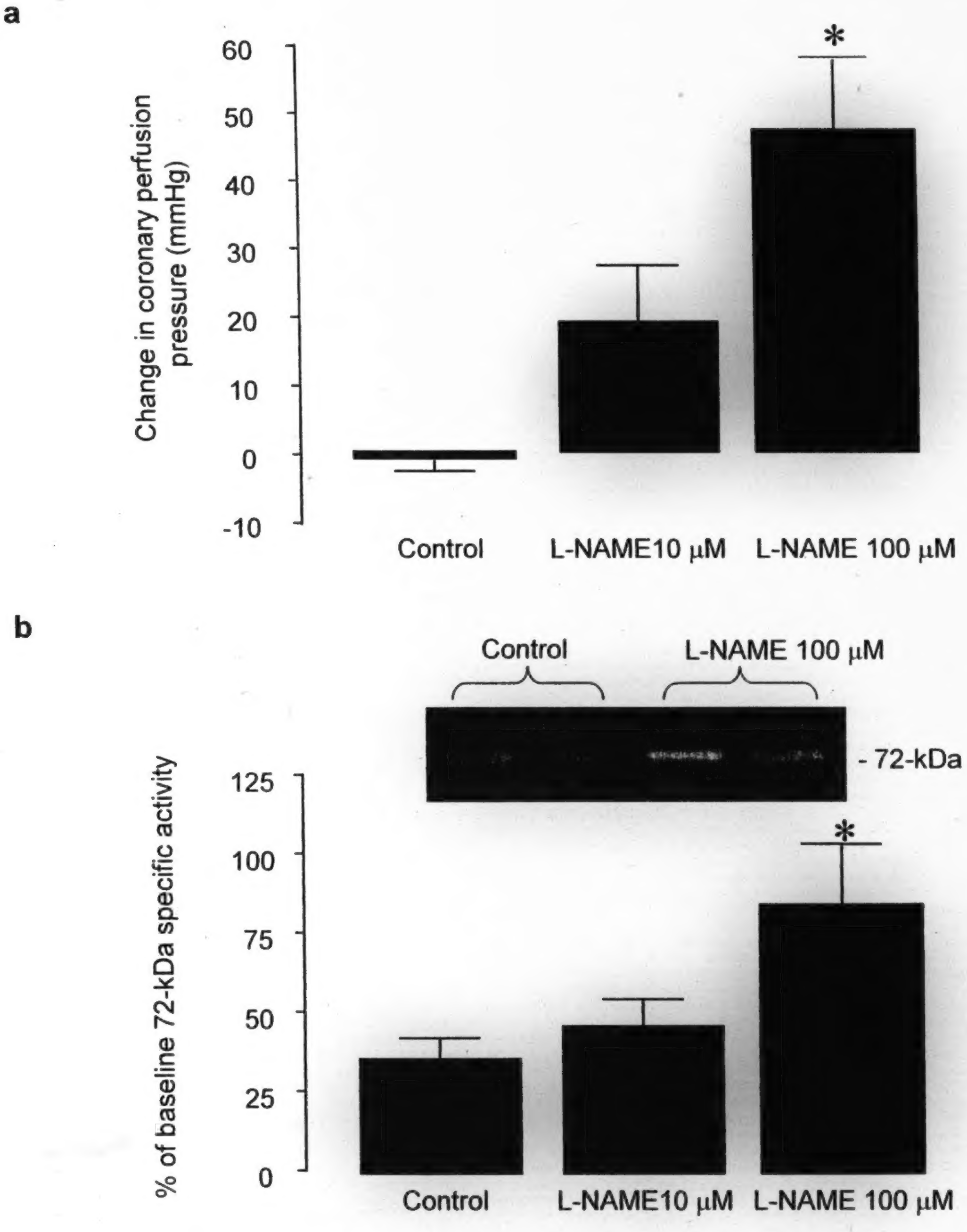

Figure 4.1 Effects of L-NAME (10 and 100 μM) on coronary perfusion pressure (a) and MMP-2 release into the coronary effluent (b) from isolated perfused rat hearts. Insert shows a representative zymogram of coronary effluent samples obtained from two control and two L-NAME infused hearts.
* $p < 0.05$ vs. control group, n = 5-8 per group

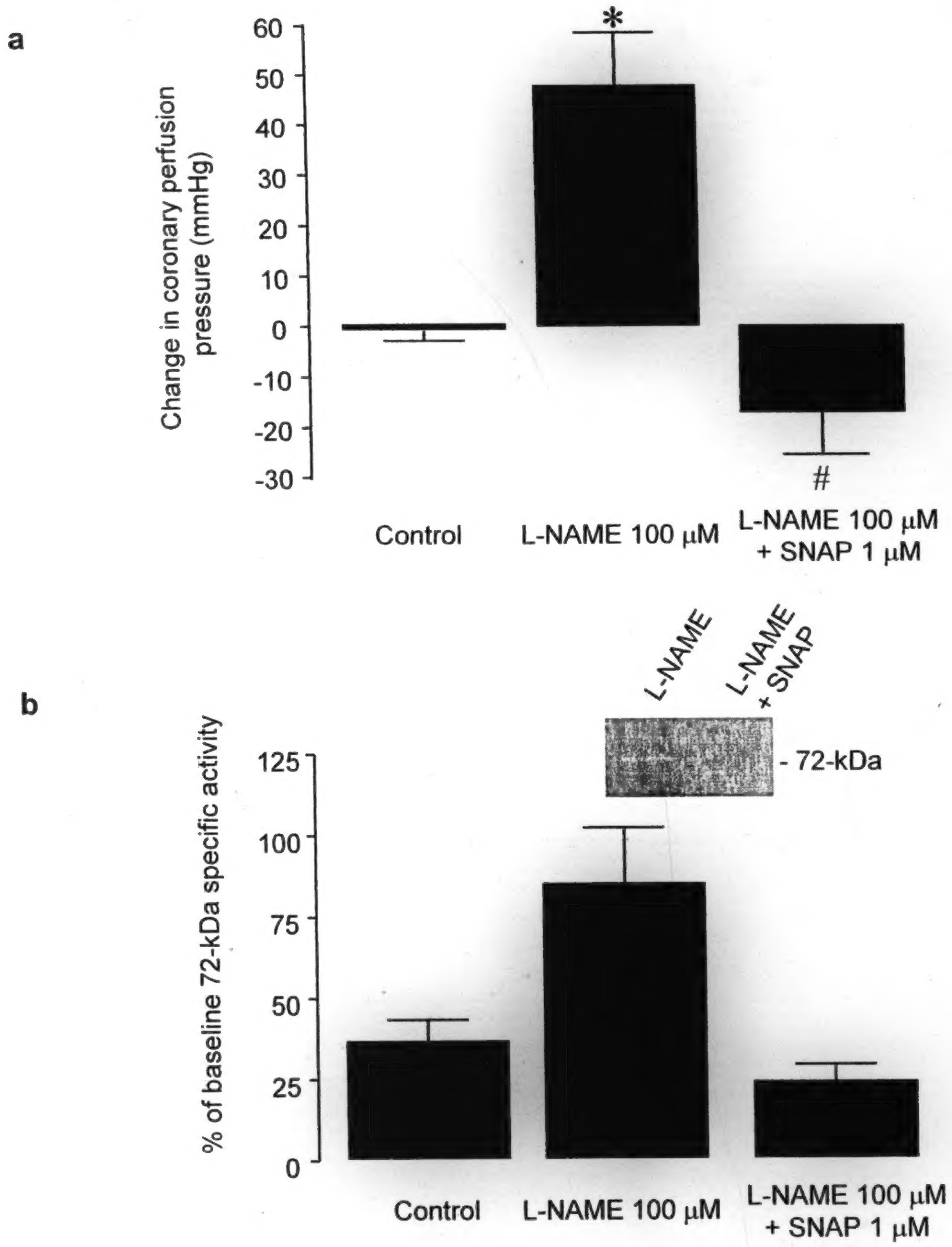

Figure 4.2 Effects of co-infusion of SNAP (1 μM) with 100 μM L-NAME on coronary perfusion pressure (a) and MMP-2 release into coronary effluent (b). Insert is a representative zymogram from coronary effluent samples. *$p < 0.05$ vs control group and # $p<0.05$ vs L-NAME group, $n = 6$-8 in each group

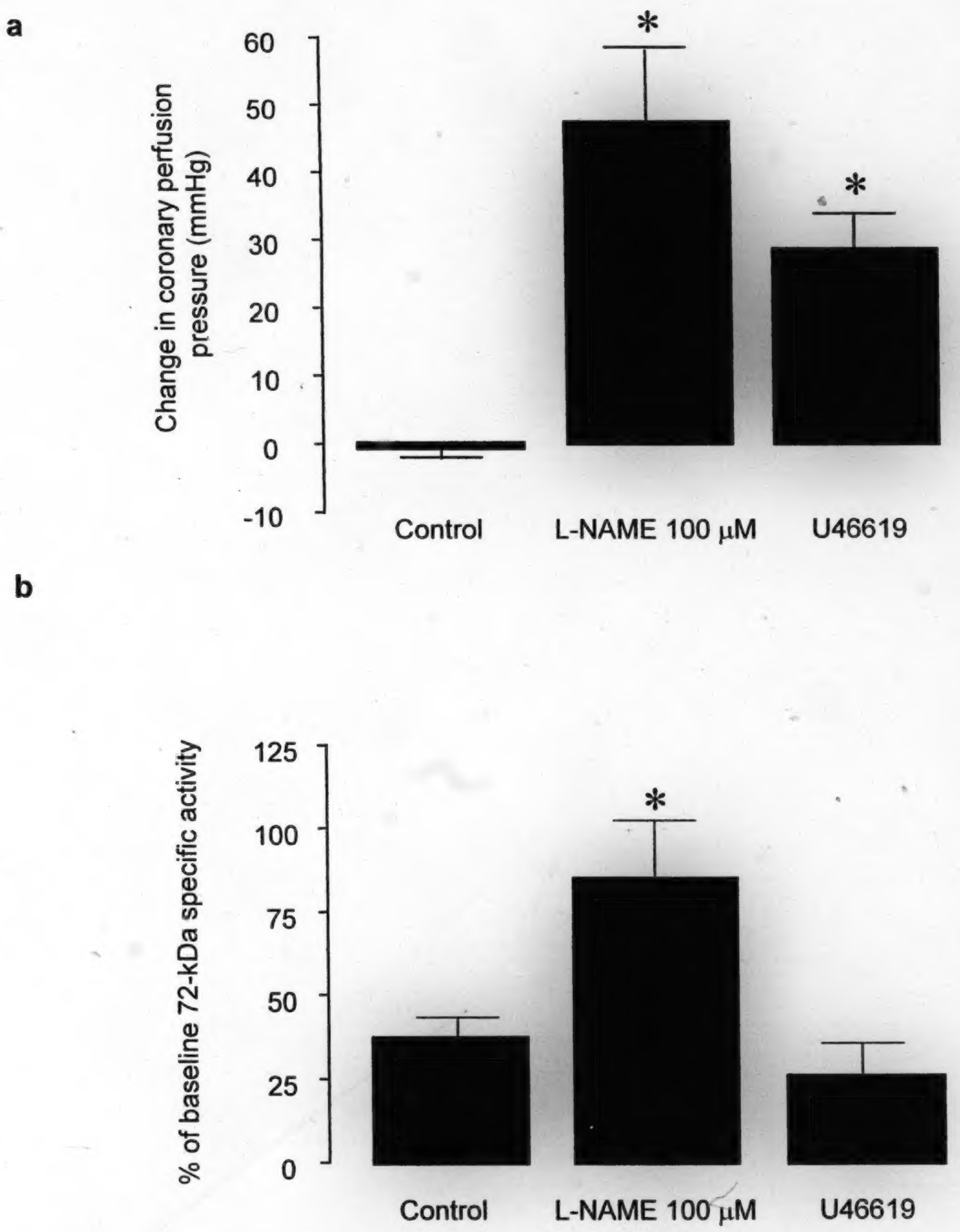

Figure 4.3 Effect of U46619 infusion on changes of coronary perfusion pressure (a) and MMP-2 release into coronary effluent (b) in comparison to that of 100 μM L-NAME in isolated perfused rat hearts. U46619 was infused at a concentration to give a statistically equivalent increase in coronary perfusion pressure (37.8 ± 14.5 nM). $^*p < 0.05$ vs control group, n = 8

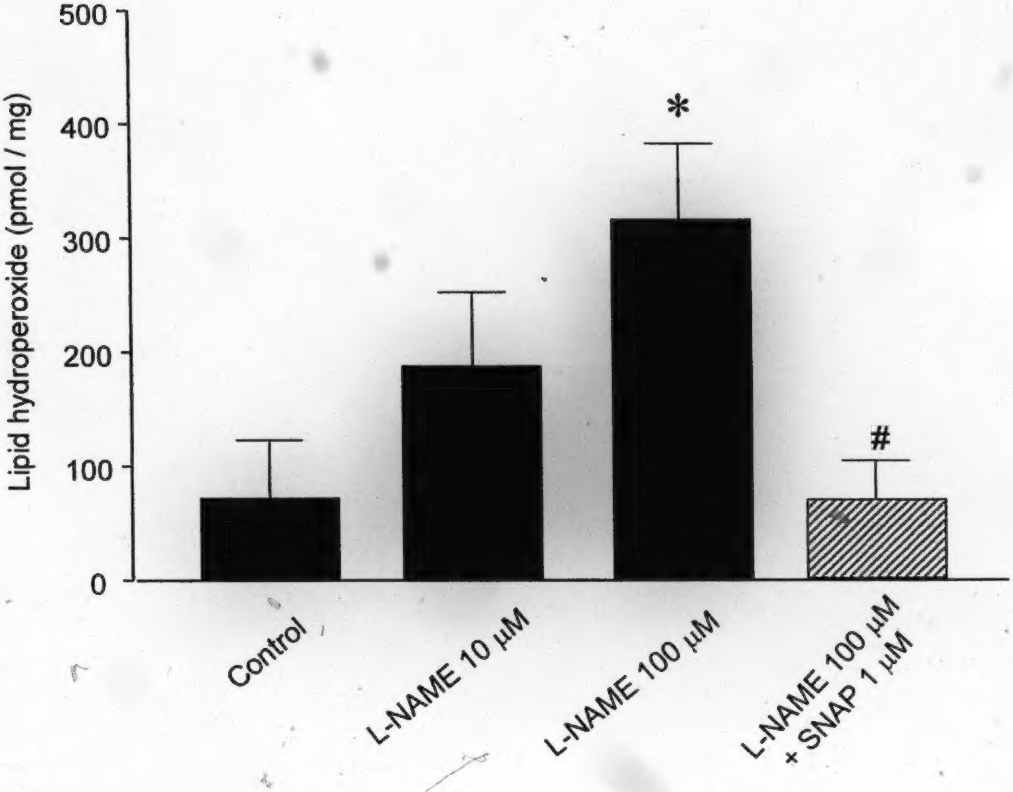

Figure 4.4 Effects of L-NAME and the co-infusion of SNAP on lipid hydroperoxide level in the myocardium. $*p < 0.05$ vs control group, n = 6 and # $p < 0.05$ vs L-NAME 100 μM group, n=5

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CHAPTER V

THE IMBALANCE BETWEEN TISSUE INHIBITOR OF METALLOPROTEINASE-4 AND MATRIX METALLOPROTEINASES DURING ACUTE MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

A version of this chapter will be submitted for publication. Costas Schulze, Wenjie Wang, Wilma L. Suarez-Pinzon, Grzegorz Sawicki & Richard Schulz. The imbalance between tissue inhibitor of metalloproteinase-4 and matrix metalloproteinases during acute myocardial ischemia-reperfusion injury. Circulation.

5.1 Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidases. They are best known for their actions in the long term remodeling of the extracellular matrix in tissue during various physiological and pathological conditions, such as embryonic development, inflammation, and cancer [1]. Recently, a number of acute, non-extracellular matrix related actions of MMPs have been described which indicate their roles in platelet aggregation [2], the regulation of vascular tone [3,4] and inflammation [5]. We recently demonstrated that MMP-2 contributes to acute myocardial ischemia-reperfusion injury [6]. In determining the locus of action of MMP-2 in the heart We also found that MMP-2 is found within the cardiac myocyte in close association with the sarcomeres[7]. MMP-2 in fact was found to be co-localized with troponin I, a key regulatory element of the actin-myosin ATPase, and contributes to its proteolytic degradation in acute myocardial ischemia-reperfusion injury[7].

MMP activity is tightly controlled by endogenous inhibitors such as the tissue inhibitors of metalloproteinase (TIMPs) [8]. Four TIMPs have been identified to date, of which TIMP-4 is the most abundant TIMP in human [9] and murine hearts [10]. Alteration in the balance between MMP and TIMP, resulting in enhanced MMP activity has been shown to occur in long-term remodeling processes such as infarction [11], heart failure [12] and dilated cardiomyopathy [13]. The role of TIMPs during acute myocardial ischemia-reperfusion is not clear. We hypothesized that an imbalance between MMP and TIMP leads to increased MMP activity during myocardial ischemia-reperfusion injury.

5.2 Materials and methods

5.2.1 Heart preparation and perfusion

Hearts were perfused at constant pressure of 60 mmHg. See section 2.2 in Chapter II for details.

5.2.2 Ischemia-reperfusion protocol

See section 2.3.3 in Chapter II.

5.2.3 Concentration of proteins in the coronary effluent

See section 2.4 in Chapter II.

5.2.4 Preparation of heart extracts

See section 2.5 in Chapter II.

5.2.5 Western blot analysis of TIMP-4

TIMP-4 content in the coronary effluent, myocardium and thin filament preparations was detected by immunoblot analysis. Samples were applied to 15% SDS-PAGE and transferred to a polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). TIMP-4 was identified using a polyclonal rabbit anti-human TIMP-4 antibody (0.25 μg/ml, Chemicon International, Temecula, CA) and band densities were measured using a HP6100 scanner (Hewlett-Packard, Boise, Idaho) and Sigmagel measurement software (Jandel, San Rafael, CA). A recombinant rat TIMP-4 protein was used as a standard (a kind gift from Dr S.C. Tyagi, University of Mississippi Medical Center, Jackson, MS).

5.2.6 *Electron microscopy with immunogold labeling*

See section 2.8 in Chapter II.

5.2.7 *Reverse zymography*

The levels of TIMPs in the coronary effluent samples were determined by reverse zymography. Briefly, concentrated coronary effluent samples were mixed with non-reducing sample loading buffer and applied to 12% polyacrylamide gel copolymerized with 1 mg/ml gelatin (Sigma, St. Louis, MO) and 50 ng/ml human recombinant MMP-2 (Oncogene, Cambridge, MA). 1-2 μg of effluent concentrate was loaded in each lane. After electrophoresis, gels were rinsed in 2.5% Triton X-100 (3× 20 min) to remove SDS. Then the gels were washed twice in incubation buffer for 20 min each at room temperature. The composition of the incubation buffer was (in mM): Tris-HCl (50), $CaCl_2$ (5), NaCl (150) and 0.05% NaN_3. The gels were then kept in incubation buffer at 37°C for 48 hr. After incubation, gels were stained in staining solution (2% Coomassie Brilliant blue G, 25% methanol, 10% acetic acid) for 2 hr and then destained twice for 30 min each in destaining solution (2% methanol, 4% acetic acid). The TIMP inhibitory activity was visualized as dark band against a clear background.

5.2.8 *Isolation of thin myofilaments*

See section 2.9 in Chapter II.

5.2.9 *In situ zymography*

In situ zymography analysis was used to determine the net tissue gelatinolytic activity from 75 min aerobically perfused and 20′ I/R hearts as described[14]. In short, left ventricles were trimmed and frozen-embedded in Cryomatrix™ (Shandon, Pittsburgh, PA) at the freezing temperature of propanol. Four μm sections were prepared using a cryostat. Sections were incubated with 0.5 mg/ml gelatin-Oregon green (Molecular Probes, Eugene, OR) in 50 mM Tris-HCl (150 mM NaCl, 5 mM $CaCl_2$) at 37 °C for 3 hr. Parallel slides with consecutive sections were incubated with the addition of 10 mM EDTA in order to inhibit MMP activity. EDTA was used because other MMP inhibitors such as o-phenanthroline interfere with the fluorometric assay. Then the sections were fixed in 1% paraformaldehyde with 0.5% D-L-glucose. Sections were examined under a Nikon-Diaphot fluorescent microscope (450-550 nm). A green color indicated positive gelatinolytic activity.

5.3 Results

5.3.1 Cardiac mechanical function recovery and release of TIMP-4 into coronary effluent by 20′ I/R hearts

20′ I/R hearts showed a depressed recovery of cardiac mechanical function compared to control hearts perfused aerobically for 75 min (Figure 5.1, 27 ± 8 % vs 84 ± 3 %, $p < 0.05$, n=6). Reverse zymography of the coronary effluent from aerobically perfused hearts demonstrated that there was a 23-kDa band corresponding to TIMP-4 (Figure 5.2a). There was no evidence of TIMP-1, -2 or -3 activity. Following 20 min of ischemia, there was a markedly enhanced release of TIMP-4 into the coronary effluent in the first min of reperfusion which was maintained over the first 5 min and which then declined to baseline levels at 10 min of reperfusion (Figure 5.2a). This TIMP-4 activity, as shown by reverse zymography, was verified to be TIMP-4 by western blot analysis (Figure 5.2b). There is no discernible change in levels of TIMP-4 in heart extracts between Aerobic 75′ and 20′ I/R groups (data not shown).

5.3.2 Duration of ischemia, release of TIMP-4 and recovery of cardiac mechanical function

We next determined the relationship between the duration of ischemia and the release of TIMP-4 into the coronary effluent in the first minute of reperfusion and how this correlates to the recovery of mechanical function. In accordance with our previous report[6], hearts subjected to 15 min ischemia followed by 30 min reperfusion showed no significant impairment in mechanical function. However, both 20 min and 25 min ischemia resulted in a significant decline in the recovery of mechanical function (Figure 5.3a). While 15′ I/R did not enhance the release of TIMP-4 above baseline release, both 20 and 25 min ischemia caused a significantly enhanced release of TIMP-4 into the coronary effluent (Figure 5.3b). There was a significant negative linear correlation between the recovery of cardiac mechanical function and the release of TIMP-4 in the first minute of reperfusion ($r = -0.58$, $p< 0.05$, $n=13$).

5.3.3 Association of TIMP-4 with sarcomeres in aerobically perfused hearts

We previously found that MMP-2 colocalized with the contractile protein regulatory element troponin I in the sarcomeres of cardiomyocytes[7]. We therefore determined the subcellular localization of TIMP-4, an inhibitor of MMP activity. Immunogold electron micrographs show a close association of TIMP-4 with sarcomeres in aerobically perfused rat hearts (Figure 5.4). There was no positive staining in sections incubated with unrelated IgG. This pattern of positive staining was reduced in 20′ I/R hearts (Figure 5.4). Two hearts from either the aerobically perfused or 20′ I/R groups were stained and 10 pictures of different sections from each heart were randomly chosen. The sarcomere-associated TIMP-4 positive staining was quantified by two independent observers in a blinded fashion. TIMP-4 staining was significantly less in sections from 20′ I/R hearts compared to Aerobic 75′ hearts (29 ± 6 vs 74 ± 9 sarcomere-associated counts per field, $p<0.05$).

5.3.4 *Loss of TIMP-4 in thin myofilament preparations from 20′ I/R hearts*

To further investigate the association of TIMP-4 with sarcomeres, highly purified thin myofilament fractions were prepared from both aerobically perfused control hearts and 20′ I/R hearts. The purity of these myofilament preparations was demonstrated by Coomassie blue staining, which showed bands for actin, tropomyosin and troponin I (Figure 5.5a). TIMP-4 immunoblots of these preparations revealed an approximately 25-kDa band in the thin myofilament preparation from aerobically perfused hearts. Pre-absorption of the anti-TIMP-4 antibody with recombinant TIMP-4 abolished its ability to recognize the 25-kDa band (Figure 5.5b). The 25-kDa band was not visible in thin myofilaments prepared from 20′ I/R hearts (Figure 5.5c).

5.3.5 Increased gelatinolytic activity in 20′ I/R hearts

With the loss of TIMP-4 from the myocardium as a result of ischemia and reperfusion, we determined whether this was associated with an increase in tissue gelatinolytic activity. Tissue sections prepared from aerobically perfused control hearts showed very little gelatinolytic activity as determined by in situ zymography (Figure 5.6). The intensity of the activity was greatly enhanced in hearts subjected to 20′ I/R. This gelatinolytic activity was significantly reduced in the presence of 10 mM EDTA, a blocker of metalloproteinase activity.

5.4 Discussion

We report here for the first time that ischemia and reperfusion injury of the heart results in the rapid loss of TIMP-4 from the heart into the coronary effluent. This release of TIMP-4 was dependent upon the duration of ischemia and was significantly enhanced only when the duration of ischemia was long enough to cause a reduced recovery of mechanical function. We also found that TIMP-4 was localized to sarcomeres in the normal heart. Subjecting the heart to ischemia and reperfusion resulted in a loss of TIMP-4 from the thin myofilament fraction. The loss of this protein, which inhibits MMP activity, was associated with an enhanced gelatinolytic activity in the myocardium during reperfusion. These results suggest that TIMP-4 plays an important homeostatic role in the normal myocardium and that its release from the heart contributes to myocardial ischemia-reperfusion injury.

We have previously shown that that there is also a rapid release of MMP-2 from the ischemic-reperfused heart using this same model and that pharmacological inhibitors of MMP-2 activity protect the heart from ischemia-reperfusion injury (see Chapter VI)[7]. We discovered that MMP-2 co-localizes with the thin myofilament regulatory protein troponin I (TnI) in the normal heart and contributes to the degradation of TnI. The degradation of TnI accounts for diminished contractile function as a result of ischemia-reperfusion injury in rat[15] and human[16] but not pig[17,18] hearts. During ischemia-reperfusion injury, there was an accumulation of MMP-2 within the thin myofilaments (see Chapter VI). This observation led us to consider whether TIMP-4 may also be associated with the thin myofilaments. Here we report that there is a loss of TIMP-4 associated with the thin myofilaments during acute myocardial ischemia-reperfusion injury. The enhanced gelatinolytic activity we observed suggests that there is an imbalance between TIMP-4 and MMP-2 and thus increased MMP activity in the myocardium.

Four isoforms of TIMPs have been characterized, namely, TIMP-1, -2, -3 and -4. They share a high sequence homology but distinct characteristics[19]. TIMP-4 is predominantly expressed in the heart compared to other organs[9]. We verified that there is a high expression of TIMP-4 in rat hearts by western blot analysis. We found no evidence of other TIMPs in these hearts. Besides its inhibitory action on MMPs, the physiological function of TIMP-4 and the reason why it is selectively expressed in the heart are not well understood. Tummalapalli *et al.* suggested that TIMP-4 expression in cardiac fibroblasts regulates their normal cell phenotype[20]. Here we identified the association of TIMP-4 in thin myofilament preparations from aerobically perfused rat hearts. The somewhat higher molecular weight of the TIMP-4 band in the thin myofilament preparations may be due to an unprocessed 29 amino acid leader sequence[19]. Several independent reports demonstrate that TIMP-4, unlike TIMP-2, does not promote but actually inhibits the activation of pro-MMP-2[21,22]. Our data suggest that there is a protective role of TIMP-4 in the normal heart.

The imbalance between TIMPs and MMPs has been suggested in the long term remodeling processes such as myocardial infarction[11], heart failure[12] and dilated cardiomyopathy[13]. All these studies showed that TIMPs are reduced in the diseased hearts while MMPs are upregulated over days or months. Here we report that a rapidly enhanced release of TIMP-4 from the hearts in the first minute during reperfusion following ischemia. The loss of TIMP-4 from myofilaments was a consequence of this release. Electron micrographs labeled with anti-TIMP-4 antibody demonstrated less staining in ischemic-reperfused hearts compared to those which were aerobically perfused. Combined with our previous results of an increase MMP-2 activity associated with myofilament preparations after ischemia-reperfusion (see chapter VI), it is reasonable to speculate that there is an increased intracellular net MMP activity in ischemic-reperfused hearts. This was partially confirmed by in situ zymography which is able to detect tissue net gelatinolytic activity. Compared to aerobically perfused hearts, ischemic-reperfused hearts showed a higher gelatinolytic activity which is inhibited by EDTA, suggesting that this gelatinolytic activity is indeed due to MMP activity (Figure 5.6).

Our results are in good accordance with findings by Mayers *et al.* in biopsies of human myocardium from patients undergoing cardiopulmonary bypass surgery which an increased net gelatinolytic activity after ischemia-reperfusion, something which is now documented in the current study using in situ zymography.

Acute ischemia-reperfusion of the heart is not a rare situation in clinical practice. How to prevent post-ischemic cardiac dysfunction is a major challenge for physicians. Here we present a new pathway leading to a depressed cardiac function in the acute phase. In summary, we report that TIMP-4 is expressed in normal myocardium in close association with sarcomeres. During reperfusion after ischemia, there is a rapid release of TIMP-4 into the coronary effluent. Recovery of cardiac mechanical function after ischemia-reperfusion negatively correlates with the amount of TIMP-4 released. Restoring the imbalance between TIMPs and MMPs might be a useful strategy in the treatment of acute cardiac ischemic syndrome. For example, MMP inhibitors and/or targeted gene delivery of TIMPs could be very promising strategies.

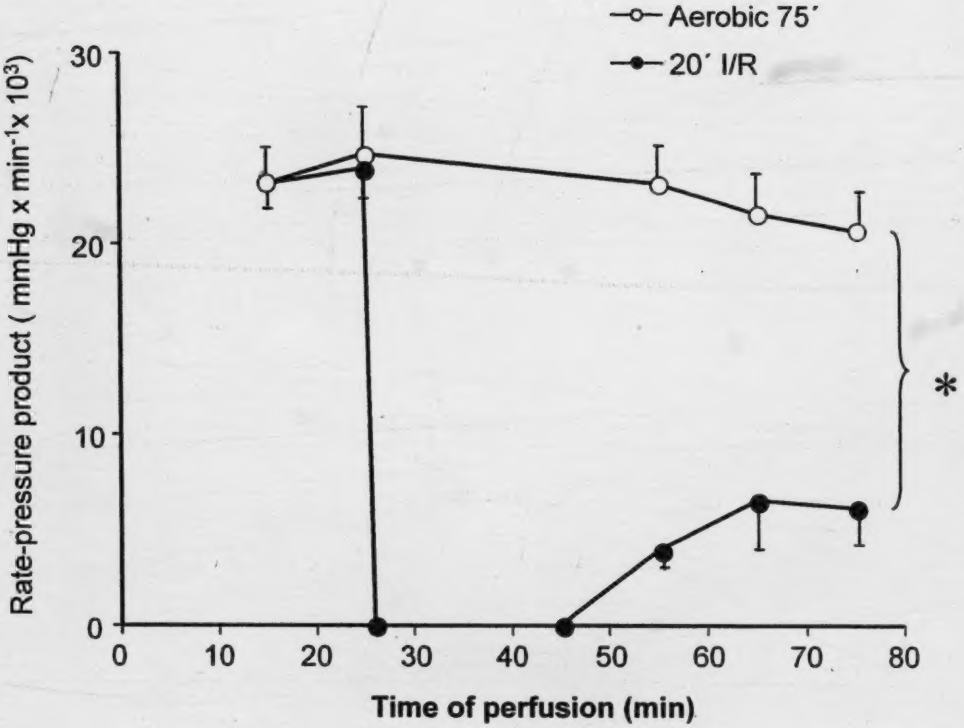


Figure 5.1 Cardiac mechanical function (rate-pressure product) of isolated rat hearts subjected to ischemia and reperfusion (20' I/R). * $p<0.05$ vs control hearts aerobically perfused for 75′ by two-way ANOVA, n=6-7.

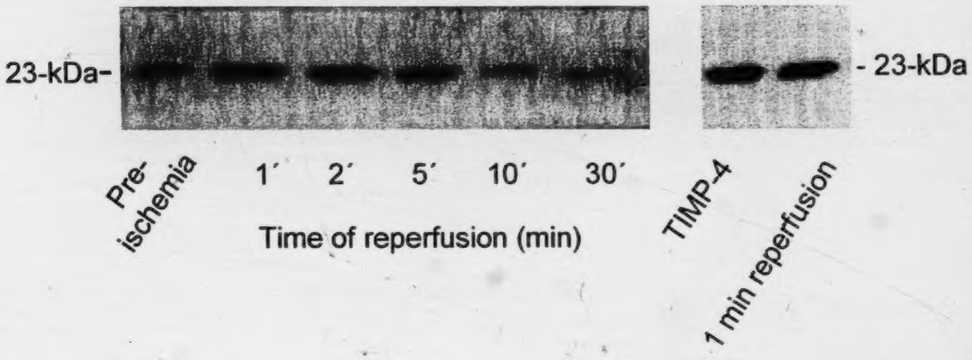

23-kDa-
- 23-kDa
Pre-ischemia
1′
2′
5′
10′
30′
Time of reperfusion (min)
TIMP-4
1 min reperfusion

Figure 5.2 Reverse zymographic analysis of coronary effluent samples from hearts subjected to 20′ I/R (left). Pre-ischemia means samples collected during aerobic perfusion immediately before ischemia. This is a representative figure from one heart perfusion with similar results seen in 5 hearts. Western blot analysis of a coronary effluent sample taken during the first minute of reperfusion after ischemia (right).

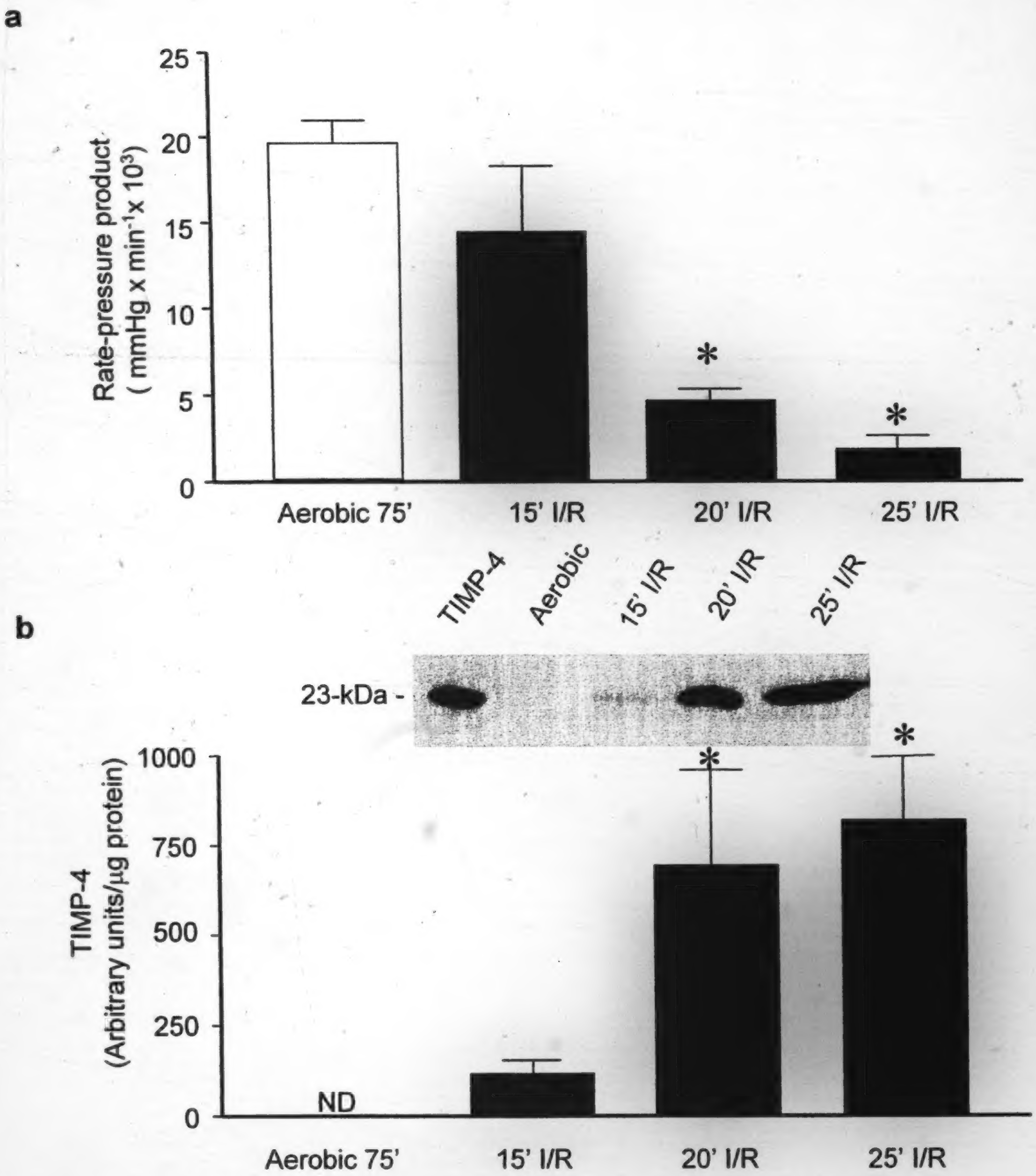


Figure 5.3 (a) Cardiac mechanical function of hearts subjected to different durations of ischemia. Hearts were aerobically perfused for 75 min (Aerobic 75′) or subjected to either 15, 20 or 25 min ischemia followed by 30' reperfusion (15′ I/R, 20′ I/R and 25′ I/R respectively). (b) Densitometric analysis of TIMP-4 western blots of coronary effluent samples taken during the first min of reperfusion from hearts subjected to different durations of ischemia. Insert is a representative western blot. ND denotes not detectable. * $p < 0.05$ vs Aerobic 75′ by one-way ANOVA with Dunnett's Multiple Comparison Test.

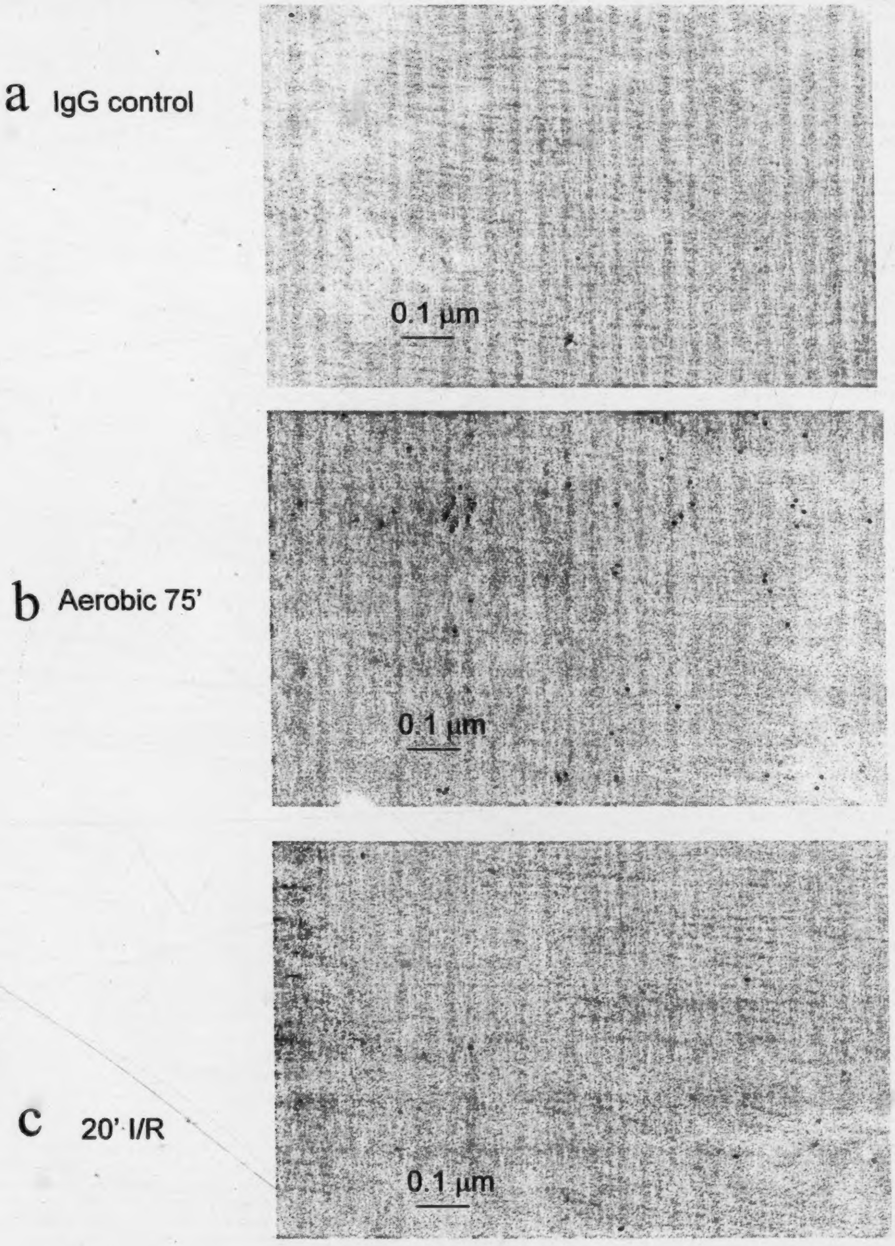

a IgG control
0.1 µm
b Aerobic 75'
0.1 µm
c 20' I/R
0.1 µm

Figure 5.4 Immunogold electron micrographs. (a) Isotype IgG control staining, (b) control hearts aerobically perfused for 75′ and (c) 20′ I/R hearts stained with anti-TIMP-4 antibody. Black dots indicate TIMP-4 staining. Magnification is ×72,000.

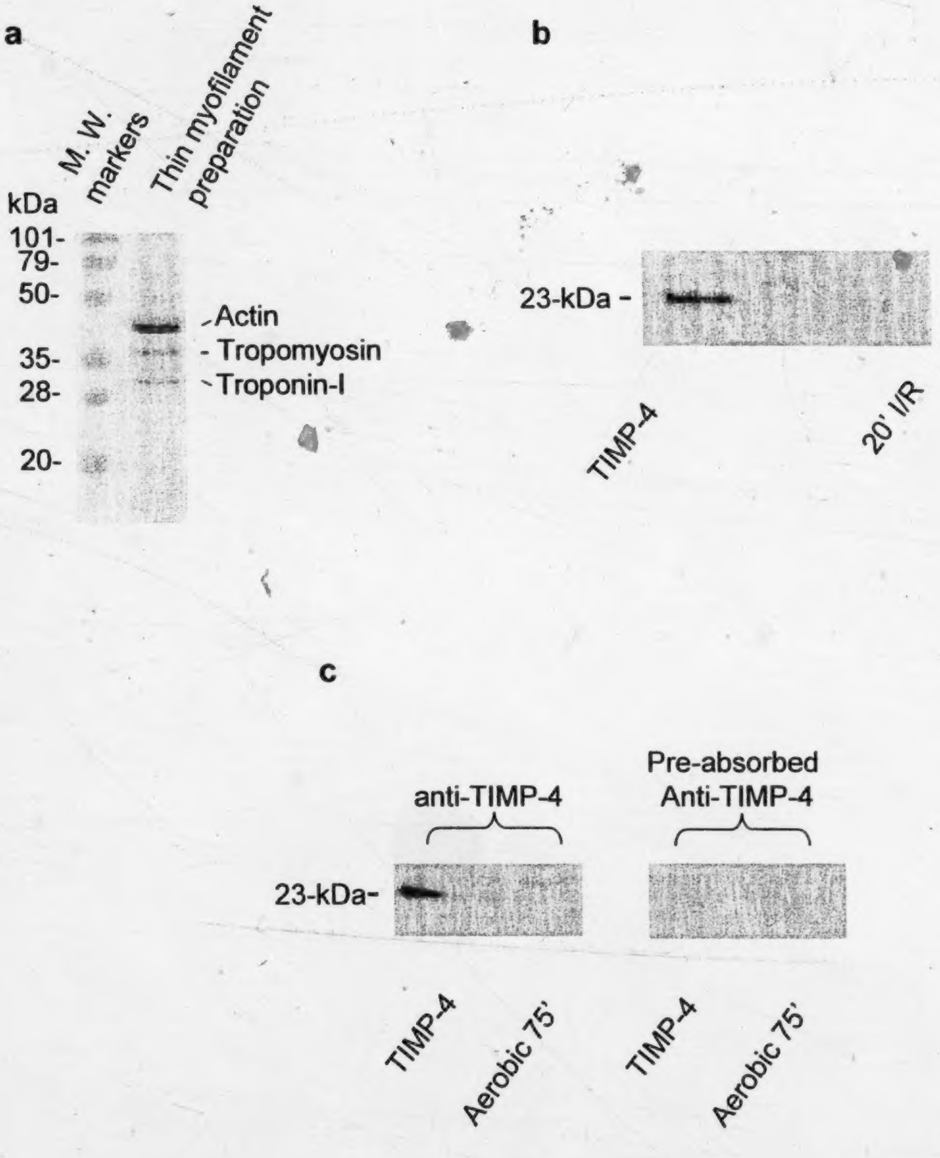
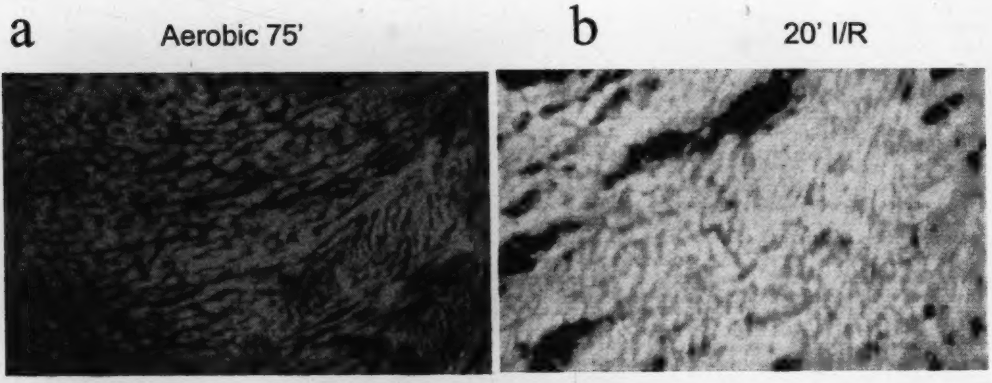
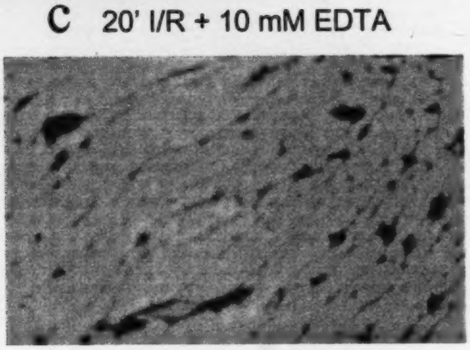

a
M. W. markers
Thin myofilament preparation
kDa
101-
79-
50-
35-
28-
20-
Actin
Tropomyosin
Troponin-I
b
23-kDa -
TIMP-4
20' I/R
c
anti-TIMP-4
Pre-absorbed Anti-TIMP-4
23-kDa-
TIMP-4
Aerobic 75'
TIMP-4
Aerobic 75'

Figure 5.5 (a) Coomassie blue stain showing the purity of a thin myofilament fraction prepared from three 20′ I/R hearts. (b) Western blot analysis of thin myofilament preparations from aerobic 75′ and 20′ I/R hearts. This is representative blot from three independent thin myofilament preparations which showed similar result. (c) Western blot analysis of thin myofilaments prepared from aerobic 75′ hearts using rabbit anti-human TIMP-4 antibody (left) or rabbit anti-human TIMP-4 pre-incubated with recombinant rat TIMP-4 (right).



a Aerobic 75'

b 20' I/R



c 20' I/R + 10 mM EDTA

Figure 5.6 In situ zymography analysis showing gelatinolytic activity of sections from: (a) a heart aerobically perfused for 75 min (Aerobic 75'), (b) a heart subjected to 20 min ischemia followed by 30 min reperfusion (20' I/R) and, (c) a 20' I/R heart when the incubation of the slides was done in the presence of 10 mM EDTA. The staining and observation were performed as described in the *Material and Methods* section. A green color indicated positive gelatinolytic activity.

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CHAPTER VI

INTRACELLULAR ACTION OF MATRIX METALLOPROTEINASE-2 ACCOUNTS FOR ACUTE MYOCARDIAL ISCHEMIA AND REPERFUSION INJURY

A version of this chapter has been submitted for publication. Wenjie Wang, Costas J. Schulze, Wilma L. Suarez-Pinzon, Jason R.B. Dyck, Grzegorz Sawicki & Richard Schulz. Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. Circulation.

6.1 Introduction

Matrix metalloproteinases (MMPs) are involved in the degradation of extracellular matrix. They are synthesized by a variety of cells in zymogen forms (pro-MMPs) and can be activated by either proteolytic cleavage[1] or oxidative stress[2]. MMPs contribute to long-term remodeling processes such as embryogenesis, inflammation, tumor invasion, angiogenesis and wound healing[3]. However, recent studies showed that MMP-2 (also known as gelatinase A or type IV collagenase; EC3.4.24.24), has rapid effects (seconds to minutes) in regulating diverse cellular functions independent of its action on the extracellular matrix. This includes effects on platelet aggregation[4], vascular tone[5,6] and mediating the acute mechanical dysfunction of the heart immediately following ischemia and reperfusion[7].

In the heart, ischemia-reperfusion injury results in partial proteolysis of the thin filament regulatory protein troponin I (TnI)[8-11] and overexpression of a TnI degradation fragment in transgenic mice[12] or deletion of the cardiac TnI gene[13] results in a phenotype with impaired cardiac mechanical function. Proteolysis of TnI by a Ca^{2+} - activated protease calpain I[14] has been suggested to participate in the impairment of cardiac function[8]. However, activation of calpain I in the heart has only been shown to occur with prolonged ischemia[15] and a direct demonstration for its involvement in stunned myocardium is lacking[8].

In both animal[7,16-18] and human myocardium[19], there is a predominant expression of MMP-2 which is also found directly in cardiac myocytes[18]. We recently demonstrated that inhibition of MMP-2 activity improves the post-ischemic recovery of cardiac mechanical function[7]. We therefore aimed to determine the possible molecular targets of MMP-2 in mediating ischemia-reperfusion injury and hypothesized that MMP-2 is involved in the degradation of troponin.

6.2 Materials and methods

6.2.1 In vitro degradation of troponin by MMP-2

2 μg of recombinant human TnI (Sigma, St. Louis, Missouri), troponin T (TnT) or troponin C (TnC, both from Calbiochem, La Jolla, California) or 6.3 μg troponin complex (TriChem Resources, West Chester, Pennsylvania) were incubated with human recombinant MMP-2 (25-750 pg/μl, Oncogene, Boston, Massachusetts) in 50 mM Tris-HCl buffer (5 mM $CaCl_2$, 150 mM NaCl) at 37 °C for 20 min. In an additional set of experiments MMP-2 was preincubated with either human recombinant TIMP-2 (0.5 μM), doxycycline (100 μM) or *o*-phenanthroline (100 μM) for 15 min at 37 °C before adding TnI. The reaction mixtures (total volume 40 μl) were analyzed by 12% or 15% SDS-PAGE under reducing conditions and visualized by the silver staining method[20]. After further purification by high performance liquid chromatography, microsequencing or electrospray mass spectrometry analysis were performed to verify the origin of the degradation products (Alberta Peptide Institute, Edmonton, Canada).

6.2.2 Isolated heart perfusion

See section 2.2 and 2.3 in Chapter II.

6.2.3 Western blotting

TnI content in coronary effluent and myocardium and MMP-2 content in thin myofilament preparations were determined by western blot analysis[21]. 30 µl of coronary effluent concentrates or 20 µg protein from heart extracts were applied to 15% SDS-PAGE gel and transferred to a polyvinylidene difluoride membranes (Bio-Rad Lab., Hercules, California). TnI was identified using a monoclonal anti-human TnI antibody (1 µg/ml, clone 8I-7, Spectral Diagnostics, Toronto, Canada). 20 µg of protein from thin myofilament preparations was subjected to 8% SDS-PAGE followed by an identical transfer. Rabbit polyclonal anti-MMP-2 antibody (1 µg/ml, a kind gift from Dr. Mieczyslaw Wozniak, Medical University, Wrocław, Poland) was used for MMP-2 identification. Gels were scanned using a HP6100 scanner (Hewlett-Packard, Boise, Idaho) and band densities were measured with Sigmagel software (Jandel, San Rafael, California). The band density was adjusted between membranes for samples run on the same day according to the average density of two samples randomly selected, which were loaded as internal controls in both or more of the needed membranes. Results were expressed as arbitrary units per µg protein.

6.2.4 Electron microscopy with immunogold labeling

See section 2.8 in Chapter II.

6.2.5 Immunoprecipitation

Heart extracts (*n* = 5) were incubated with either mouse monoclonal IgG_1 (ICN Biomedicals, Aurora, Ohio; Sigma, St. Louis, Missouri) or anti-troponin I antibody (clone 8I-7, 10 µg/ml, Spectral Diagnostics, Toronto, Canada) in lysis buffer (50 mM

Tris-HCl, 150 mM NaCl and 1% NP-40) at 4°C overnight. Protein A Sepharose bead suspension (1:5 v:v, Sigma, St. Louis, Missouri) was added and incubated overnight at 4°C. The supernatant was discarded and the protein A Sepharose fraction was washed three times in 50 mM Tris-HCl (150 mM NaCl, 5 mM $CaCl_2$, 0.05% NaN_3) at 4°C. One part of the sample was analyzed by gelatin zymography as described[7]. The other part was split and further incubated for 12 hr at either 4 °C or 37 °C in the presence or absence of *o*-phenanthroline (100 μM). Products were examined by western blot for TnI content.

6.2.6 Co-localization of MMP-2 and TnI

A separate series of 20′ ischemia-reperfusion (20′ I/R) hearts were frozen at the end of 30 min reperfusion. Frozen 3 μm sections were prepared and fixed in 1 % paraformaldehyde, washed in pH 7.4 PBS, and stained with the primary antibodies (mouse monoclonal anti-TnI antibody to human TnI fragment, 3E3, Spectral Diagnostics, Toronto, Canada; rabbit polyclonal anti-MMP-2 antibody as above) for 12 hr at 25 °C. Appropriate control isotype IgGs were used as negative controls (Cedarlane Laboratories, Hornby, Canada). Two consecutive sections were placed on the same slide, one section was incubated with the primary antibody and the other with the corresponding control IgG. The slides were extensively washed with PBS and labeled in serial incubations with the polyclonal secondary antibodies for 2 hr at 4 °C. TnI was labeled with donkey anti-mouse AlexaFluor 488 (3 μg/ml), MMP-2 with donkey anti-rabbit AlexaFluor 594 (5 μg/ml, Molecular Probes, Eugene, OR). After extensive washing, the stained slides were treated with 1 % paraformaldehyde for 30 min at 25 °C and mounted with DAPI Vectashield (Vector, Burlingame CA). Stained slides were kept at 4 °C overnight and then images were viewed with a Zeiss LSM 510 NLO microscope (Carl Zeiss, Jena, Germany) using a PlanNeofluor 40x/1.3 oil objective. Argon (at 488 nm), HeNe (at 543 nm) and tunable Ti sapphire (at 800 nm) lasers were used for excitation (MIRA 900F Coherent Laser Group; Santa Clara, CA). Triple fluorescence emission images were collected with multitracking scanning using barrier filters for AlexaFlour 488-green (505 nm long pass), AlexaFlour 594-red (560 nm long pass) and DAPI-blue (685 nm short pass).

6.2.7 Isolation of thin myofilaments

See section 2.9 in Chapter II. Gelatin zymography and western blot analysis were then performed to determine MMP activity and protein content.

6.3 Results

6.3.1 In vitro degradation of troponin subunits and troponin complex

We tested purified human recombinant troponin complex components individually for their susceptibility to proteolytic degradation by MMP-2. Both TnI and TnT, but not TnC were degraded by MMP-2 in a concentration-dependent manner (Figure 6.1). TnI degradation was complete within 20 min of incubation at 37 °C with MMP-2, whereas there was only partial degradation of TnT. Similarly, TnC was found to be resistant to serine protease degradation whereas TnI and TnT were susceptible[22]. Further analysis of the proteolytic fragments by microsequencing or electrospray mass spectrometry confirmed that they were derived from TnI and TnT respectively. In TnI three cleavage sites include $P^{17}I^{18}$, $N^{24}Y^{25}$ and $A^{156}L^{157}$. The only identified cleavage site in TnT was $N^{179}M^{180}$. Inhibition of MMP-2 activity with the tissue inhibitor of matrix metalloproteinase-2 (TIMP-2), doxycycline or *o*-phenanthroline prevented the degradation of TnI by MMP-2 (Figure 6.1d).

We next examined whether TnI and TnT are susceptible to cleavage by MMP-2 when assembled in the intact troponin complex. Incubation of troponin complex with MMP-2 revealed that only TnI, but not TnT or TnC, was susceptible to proteolysis (Figure 6.2).

6.3.2 MMP inhibitors diminish TnI cleavage in the heart and improve mechanical function

To investigate whether MMP-2 can cleave TnI in intact myocardium under pathophysiological conditions we isolated and perfused rat hearts under one of three conditions: a) aerobic perfusion (Aerobic); b) 20 min of global, no-flow ischemia and 30 min of aerobic reperfusion (20′ I/R); or c) 20′ I/R in the presence of MMP inhibitors doxycycline or *o*-phenanthroline. The recovery of post-ischemic mechanical function at the end of the 30 min reperfusion period was significantly depressed and only 31 ± 12% of that measured in aerobically perfused hearts (Figure 6.3a). Doxycycline or *o*-phenanthroline significantly improved the recovery of function of hearts subjected to ischemia-reperfusion to 63 ± 11% and 82 ± 14% of the aerobic group, respectively (Figure 6.3a). Using an antibody against recombinant human TnI, western blot analysis of the heart tissue homogenates from aerobic hearts revealed two bands of approximately 31 kDa and 27 kDa (Figure 6.3b). 20′ I/R resulted in a marked loss in the density of the 31 kDa band, which was protected in hearts subjected to doxycycline or *o*-phenanthroline treatment (Figure 6.3b). In contrast, phosphoramidon, an inhibitor of some metalloproteinases but not of MMPs[23] neither enhanced post-ischemic mechanical function nor prevented the loss in TnI content (data not shown). TnI or its degradation products were not detectable in the coronary effluent in any of the three perfusion conditions, indicating that significant cardiomyocyte necrosis did not occur as a result of the ischemia-reperfusion injury (data not shown). These experiments suggest that MMP activity in the heart contributes to the degradation of TnI seen as a result of myocardial ischemia and reperfusion injury.

6.3.3 Infusion of MMP-2 into the heart enhances TnI degradation and worsens mechanical function

To further verify that MMP-2 was able to induce the degradation of TnI in the heart with functional consequence, we infused hearts with a preparation of MMP-2. These hearts were subjected to a shorter 15 min period of ischemia, followed by 30 min reperfusion (15′ I/R) which in itself does not result in a significant impairment of cardiac mechanical function (the rate pressure product was 25.9 ± 2.5 versus 22.4 ± 2.6 mmHg × $\text{min}^{-1} \times 10^3$ in aerobically perfused hearts, $n = 5$ per group, $P > 0.05$). In contrast, infusion of MMP-2 significantly impaired the recovery of mechanical function (46 ± 16% of 15′ I/R, Figure 6.4a) and reduced the myocardial content of TnI (65 ± 9% of 15′ I/R, Figure 6.4b).

6.3.4 Immunoprecipitation reveals an association of MMP-2 with TnI in the heart

The extract of hearts subjected to 20′ I/R was immunoprecipitated with either anti-TnI antibody or unrelated IgG_1. Immunoblot analysis of the precipitate with anti-TnI antibody, but not IgG_1, showed a 31kDa band corresponding to native TnI (Figure 6.5a). Subsequent incubation of this immunoprecipitate at 37 °C (but not 4 °C) for 12 h resulted in TnI degradation (Figure 6.5a). This degradation was abolished by *o*-phenanthroline, indicating the presence of MMP activity in the immunoprecipitate (Figure 6.5a). Further analysis of the immunoprecipitate with gelatin zymography confirmed the association of MMP-2 and pro-MMP-2 activities with TnI (Figure 6.5b).

6.3.5 Immunolocalization of MMP-2 after ischemia-reperfusion

Immunoelectron microscopy studies were performed to determine whether MMP-2 can be localized to sarcomeres where TnI is located. An anti-MMP-2 antibody was used which recognizes MMP-2 but not pro-MMP-2[7]. In 20′ I/R rat hearts extensive staining for MMP-2 occurred within the sarcomeres, in close association with the myofilaments (Figure 6.6a). Interestingly there was also some mitochondrial association of MMP-2 (Figure 6.6a). Use of isotype control IgG instead of anti-MMP-2 revealed no positive staining (data not shown). A preparation of highly purified thin myofilaments[24] from 20′ I/R hearts revealed the association of gelatinolytic activities corresponding to MMP-2 and pro-MMP-2, with the former in excess (Figure 6.6b). Thin myofilament preparations from 20′ I/R hearts showed higher MMP-2 content by western blot analysis than preparations from aerobically perfused hearts (Figure 6.6c).

20′ I/R hearts were sectioned and processed for immunohistochemistry using confocal microscopy. Multitracking scanning using barrier filters up to 20 sections (0.4 μm each in thickness) clearly showed intracellular localization of MMP-2 within cardiac myocytes (Figure 6.7). To determine whether MMP-2 is co-localized with TnI, dual label immunofluorescence using confocal microscopy was performed. The image for TnI shows, as expected, a strong immunostaining along the myofilaments (Figure 6.8). MMP-2 showed an intermittent and diffuse localization pattern which was both membrane-associated and cytoplasmic. Stained sections incubated with control IgGs for each of the primary antibodies showed no fluorescence signals (data not shown). The dual label image reveals a clear association of MMP-2 with TnI within cardiac myocytes.

6.4 Discussion

We demonstrated that MMP-2 is able to proteolytically cleave TnI. In hearts subjected to ischemia and reperfusion injury MMP-2 is localized to sarcomeres in close association with the thin myofilaments. Dual label immunofluorescence studies provide clear evidence of the co-localization of MMP-2 with TnI. Inhibition of MMP-2 activity reduced TnI degradation whereas infusion of MMP-2 enhanced it. These data show for the first time an intracellular action of MMP-2. We have thus shown that a key defect of myocardial ischemia-reperfusion injury, the degradation of TnI, is caused by MMP-2. This is a novel molecular mechanism which causes diminished contractile function of the heart.

These findings are of particular importance since MMPs have been recognized as significant contributors to other cardiac pathologies in both humans and animals including congestive heart failure[19,25], acute myocardial infarction[26] and dilated cardiomyopathy[27,28]. Interestingly MMPs were speculated to have biological effects apart from their action on the extracellular matrix[29]. Indeed protective actions of MMP inhibitors on myocardial contractile function were found to be independent of changes in collagen content[26]. An intracardiomyocyte and sarcomeric association of MMP-2 was found in hearts of patients with dilated cardiomyopathy[30].

Several lines of evidence from our data demonstrate that MMP-2 has an intracellular locus of action on TnI in mediating ischemia-reperfusion injury:

a) MMP-2 activity is found in highly purified thin myofilaments and the level of MMP-2 is enhanced following ischemia and reperfusion, b) the presence of MMP-2 activity in the anti-TnI immunoprecipitate from heart homogenates, c) immunogold electron microscopical localization of MMP-2 within sarcomeres, d) co-localization of MMP-2 and TnI using confocal microscopy, e) pharmacological prevention of TnI degradation with cell permeable MMP inhibitors (doxycycline and *o*-phenanthroline) and f) the absence of detectable TnI or its degradation fragments in the coronary effluent.

The decrease in myocardial TnI content following ischemia and reperfusion observed here (Figure 6.3) is likely a result of degradation, given the co-localization of MMP-2 with TnI in the setting of ischemia-reperfusion and the susceptibility of TnI to degradation by MMP-2. However, we cannot rule out, in addition to degradation, the occurrence of post-translational modifications of TnI which lead to diminished binding of the TnI antibody to the protein. Myocardial TnI content, however, was shown to diminish with increasing duration of ischemia followed by reperfusion[10]. Inhibitors of MMP activity or the NO donor S-nitroso N-acetylpenicillamine (data not shown), known also to be cardiopotective in ischemia-reperfusion injury[31], preserved TnI content.

MMP-2 was localized within cardiac myocytes, including along the Z lines (Figure 6.6a). As MMP-2 was also found to be co-localized with α-actinin (also found in

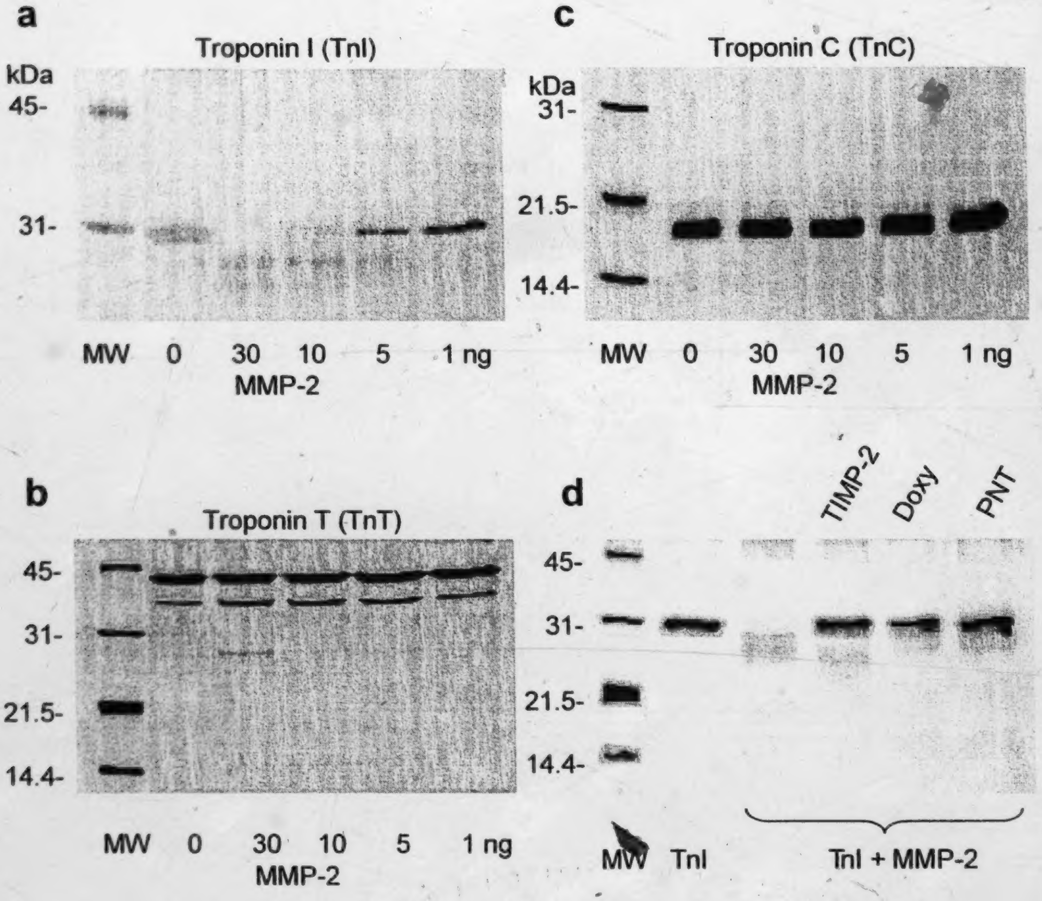
Z lines) in cardiac myocytes[18] and it is known that α-actinin degradation occurs under similar conditions of ischemia and reperfusion[32], there are likely other possible targets of MMP-2 during ischemia-reperfusion injury.

Our results partially rest on the specificity of doxycycline and o-phenanthroline as inhibitors of MMPs. Their protective actions in ischemic-reperfused hearts on the recovery of mechanical function have been mimicked by neutralizing MMP-2 antibody[7]. Both doxycycline and o-phenanthroline, but not phosphoramidon, a metalloproteinase inhibitor devoid of MMP inhibitory action, prevented the loss of TnI and improved functional recovery during reperfusion. Tetracycline class antibiotics are known to also have MMP inhibitory activity, an action independent of their antibacterial effect[33]. In surveying antibiotic usage and the risk of first time acute myocardial infarct in humans, a statistically significant risk reduction was seen only in those who had taken tetracycline class, but not in those taking any other class of antibiotics[34,35].

It has been documented that TnI and TnT are susceptible to degradation by serine proteases whereas, in contrast, TnC is resistant to proteolysis[22]. In this study we found a similar pattern of susceptibility to proteolytic attack by MMP-2 on TnI and TnT, but not TnC.

Our study is the first to show that both intracellular action and targeting of MMP to a novel substrate, TnI. TnI is the first clearly recognized intracellular MMP-2 target amongst a growing list of newly discovered substrates of MMP-2 unrelated to the extracellular matrix, each invoking a novel biological action of this matrix metalloproteinase. This includes effects of MMP-2 on vascular tone by cleaving big-endothelin to a novel vasoconstrictor peptide[5] and degrading the vasodilator calcitonin gene-related peptide[6], as well as attenuating the inflammatory response by cleaving monocyte chemoattractant protein-3 to a product which is an antagonist of receptors to this protein[36]. The term "matrix metalloproteinases" does not properly reflect the full spectrum of biological actions of these proteases. Indeed this study shows that intracellular targets should also be considered in discovering new biological roles for MMPs.

Post-translational modification of contractile proteins such as TnI resulting in its depletion is a final common pathway causing contractile dysfunction of the heart[9,12]. The direct role of MMPs in causing contractile protein alterations in other cardiac pathologies needs to be studied. Pharmacological inhibition of MMP activity represents a novel strategy for the prevention or treatment of myocardial ischemia-reperfusion injury.


a
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kDa
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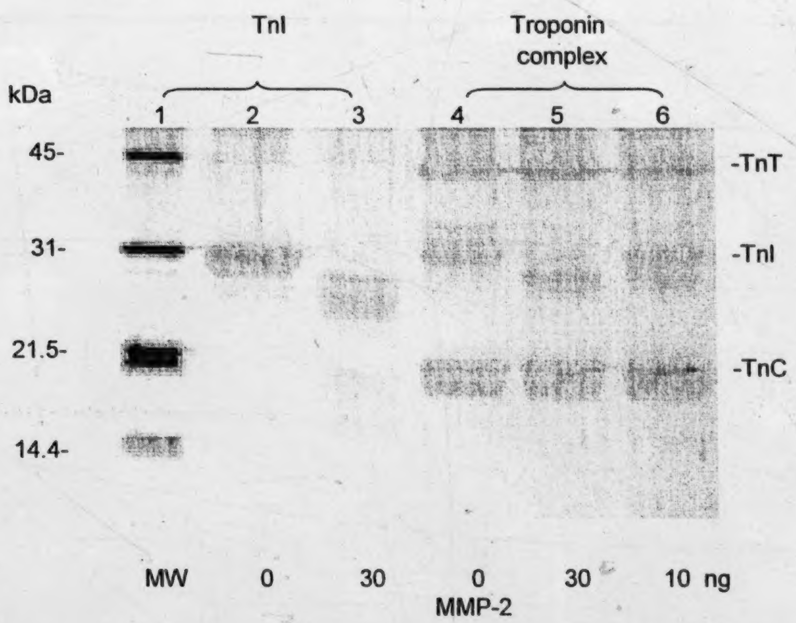
31-
MW 0 30 10 5 1 ng
MMP-2
c
Troponin C (TnC)
kDa
31-
21.5-
14.4-
MW 0 30 10 5 1 ng
MMP-2
b
Troponin T (TnT)
45-
31-
21.5-
14.4-
MW 0 30 10 5 1 ng
MMP-2
d
TIMP-2
Doxy
PNT
45-
31-
21.5-
14.4-
MW TnI
TnI + MMP-2

Figure 6.1 SDS-PAGE silver stain showing in vitro degradation of troponin I (TnI) and T (TnT) by MMP-2. MW denotes molecular weight markers. (**a**) TnI degradation as shown by decreased intensity of the 31 kDa band and appearance of additional bands at < 31 kDa. (**b**) TnT degradation. TnT preparation contained a 44 kDa mother band and an additional unknown band at ~38 kDa. TnT degradation is observed by the appearance of bands at ~29 and ~15 kDa. (**c**) TnC (18 kDa) was unaffected by MMP-2. (**d**) Inhibition of MMP-2 induced TnI degradation by MMP inhibitors TIMP-2, doxycycline (Doxy), or *o*-phenanthroline (PNT). *(with the help of C. Schulze and G. Sawicki)*


TnI
Troponin complex
kDa
1
2
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4
5
6
45-
31-
21.5-
14.4-
-TnT
-TnI
-TnC
MW
0
30
0
30
10 ng
MMP-2

Figure 6.2 SDS-PAGE silver stain showing in vitro degradation of TnI within the troponin complex by MMP-2. MW denotes molecular weight markers. Lanes 2 and 3 show TnI incubated in the absence or presence of MMP-2, respectively. Lane 4 shows troponin complex alone; 45, 31 and 18 kDa bands represent TnT, TnI, and TnC respectively. In the presence of MMP-2 degradation of TnI, but not TnT or TnC, was observed. (*with help from Costas Schulze*)

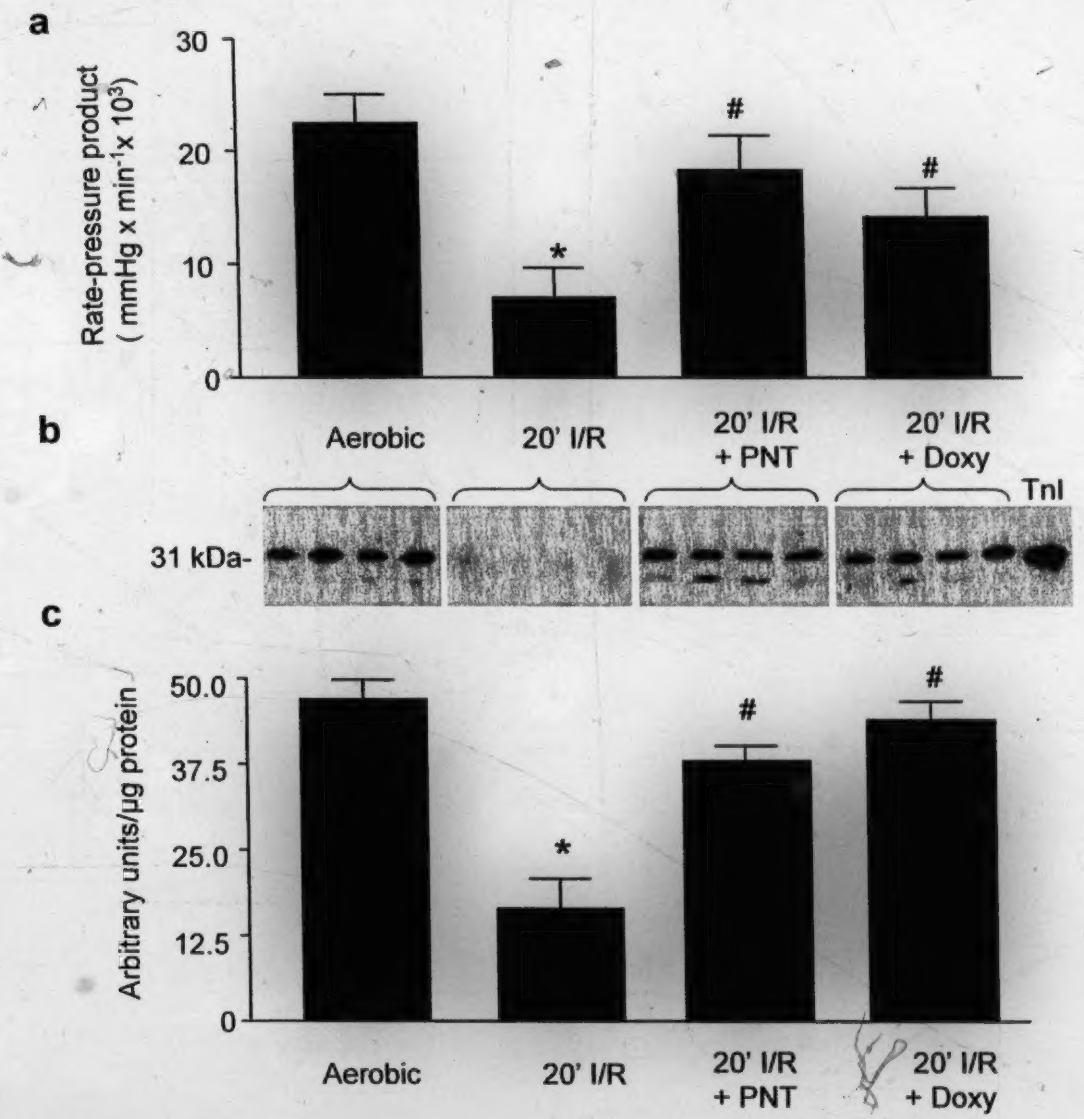

Figure 6.3 Cardiac mechanical function (rate-pressure product) of isolated rat hearts subjected to ischemia and reperfusion (20′ I/R) and its relation to myocardial TnI content. **a)** 20′ I/R hearts showed reduced recovery of mechanical function compared to hearts perfused aerobically (Aerobic). Doxy or PNT significantly improved the recovery of mechanical function. There was no significant change in heart rate in any group whereas left ventricular developed pressure at the end of reperfusion was 94 ± 8, 31 ±15, 87 ± 8, and 52 ± 9 % of pre-ischemic function in aerobic, 20′ I/R, 20′ I/R + PNT and 20′ I/R + Doxy hearts, respectively. **b)** Western blot of TnI from 4 representative hearts per group from a). 20′ I/R caused a loss of TnI in hearts which was diminished by Doxy or PNT. **c)** Densitometric analysis of TnI (31 kDa) content in heart tissue samples. n = 5-7, mean ± SEM, *P<0.05 vs. Aerobic, #P< 0.05 vs. 20′ I/R by ANOVA. *(I perfused hearts. Western blot analysis was done with the help of C. Schulze).*

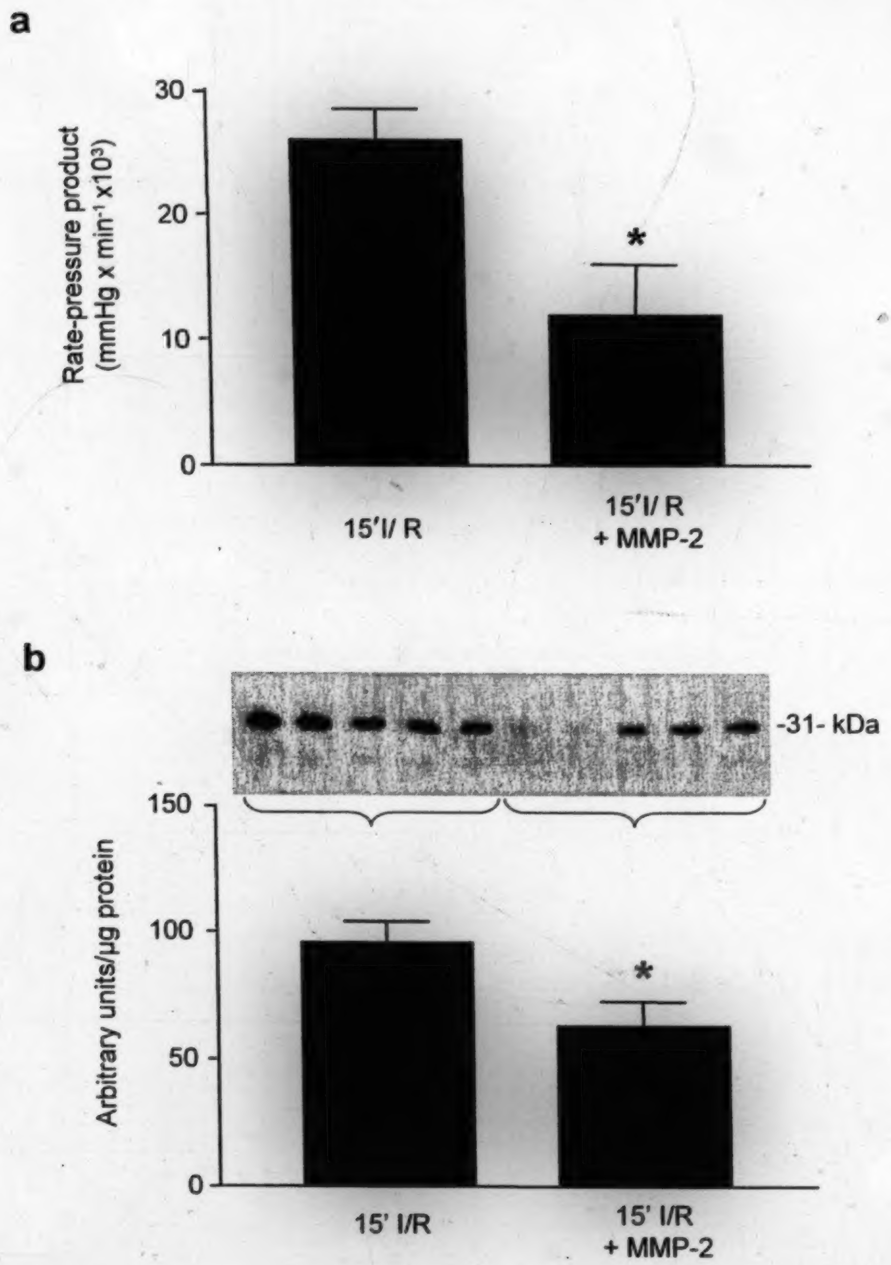

Figure 6.4 Effect of infusion of MMP-2 into isolated hearts on recovery of cardiac mechanical function after 15 min ischemia and 30 min reperfusion (15′ I/R, **a**) and densitometric analysis of myocardial TnI content determined by western blot (**b**). *n* = 5 for each group, mean ± SEM, **P*<0.05 vs. 15′ I/R by Student's *t*-test. *(I did heart perfusions. Western blot analysis was done by C. Schulze)*

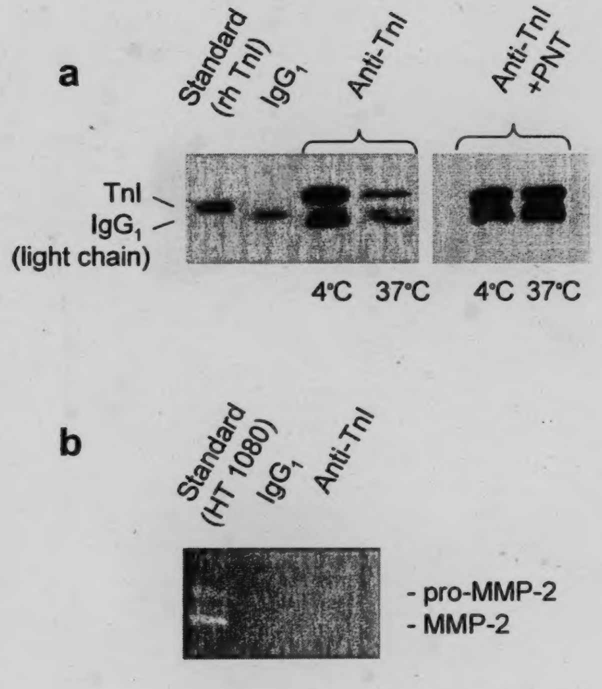
a
Standard (rh TnI)
IgG$_1$
Anti-TnI
Anti-TnI +PNT
TnI
IgG$_1$ (light chain)
4°C
37°C
4°C
37°C
b
Standard (HT 1080)
IgG$_1$
Anti-TnI
- pro-MMP-2
- MMP-2

Figure 6.5 Association of MMP-2 with TnI after ischemia-reperfusion. **(a)** Western blot analysis of TnI content in heart homogenate immunoprecipitated with either IgG_1 or anti-TnI antibody. Following immunoprecipitation with anti-TnI, the samples were split and further incubated at either 4 °C or 37 °C for 12 hr in the absence (left panel) or presence (right panel) of PNT. **(b)** Gelatin zymography analysis of heart homogenate immunoprecipitated with either IgG_1 or anti-TnI antibody. *(with the help of C. Schulze)*

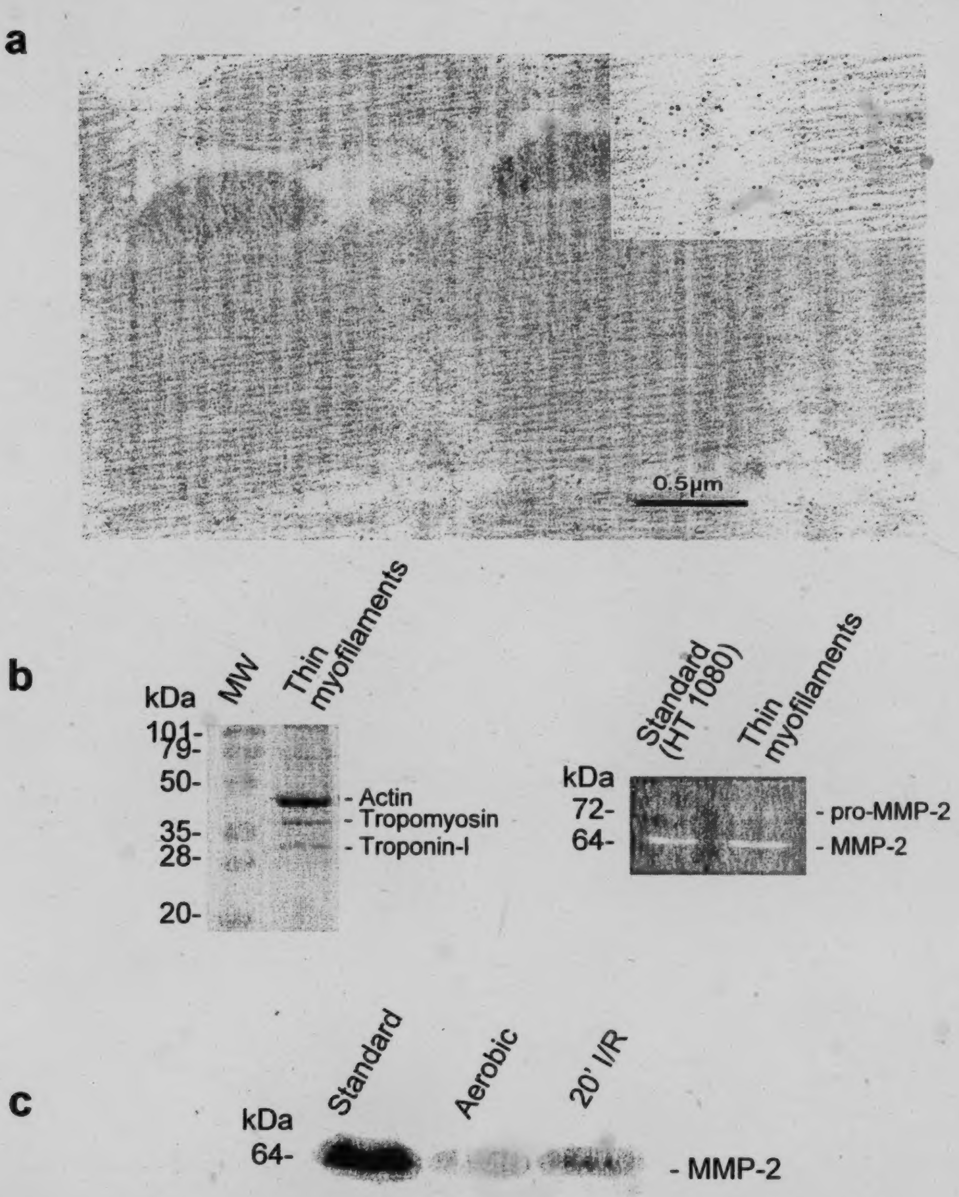
a
0.5μm
b
kDa
MW
Thin myofilaments
101-
79-
50-
35-
28-
20-
- Actin
- Tropomyosin
- Troponin-I
kDa
Standard (HT 1080)
Thin myofilaments
72-
64-
- pro-MMP-2
- MMP-2
c
kDa
Standard
Aerobic
20' I/R
64-
- MMP-2

Figure 6.6 Intracellular localization of MMP-2 in association with thin myofilaments. **(a)** Representative transmission electron micrograph of 20′ I/R hearts. Black dots indicate immunogold labeling of MMP-2 with anti-MMP-2 antibody. Magnification: 42000×; inset 72000×. **(b)** Left panel: SDS-PAGE Coomassie Blue stain of a preparation of thin myofilaments from 20′ I/R hearts. MW: molecular weight marker. Right panel: Gelatin zymography analysis of same sample showing pro-MMP-2 and MMP-2 activity associated with thin myofilaments. Standard is HT-1080 cell culture medium. **(c)** Western blot analysis of MMP-2 content in thin myofilament preparations from aerobically perfused and 20′ I/R hearts. *(with the help of C. Schulze)*

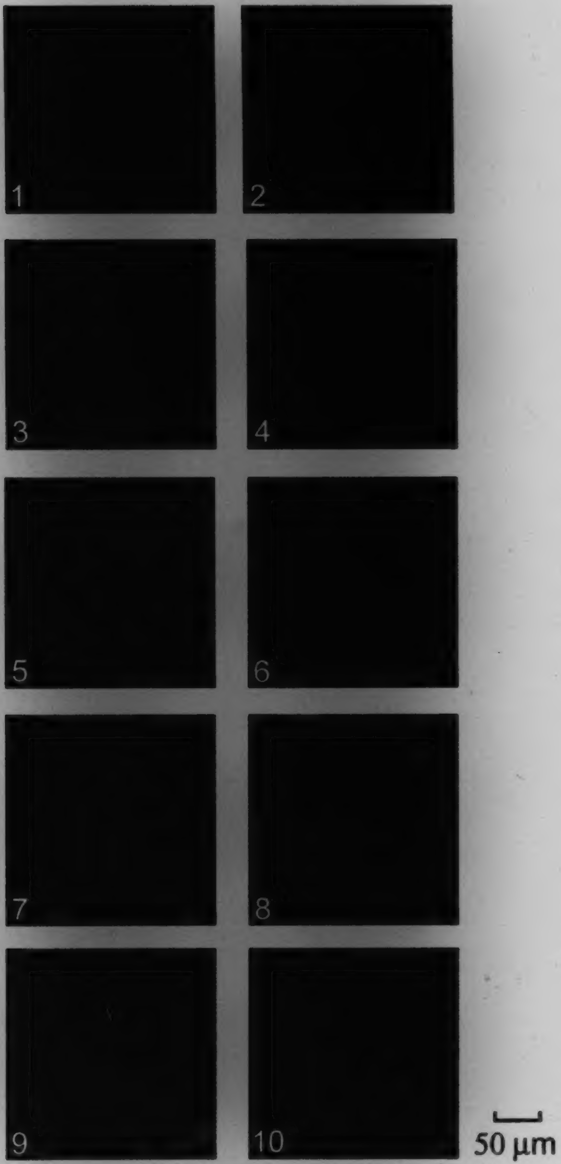

Figure 6.7 Multitracking scanning clearly showed intracellular localization of MMP-2 within cardiac myocytes. Slides were stained as described in the *Methods* section. Multitracking scanning was performed using barrier filters up to 20 sections (0.4 µm thick). Nuclei were stained with DAPI Vectashield (blue color). Red color indicates MMP-2 staining. Numbers 1-10 indicate consecutive sections.

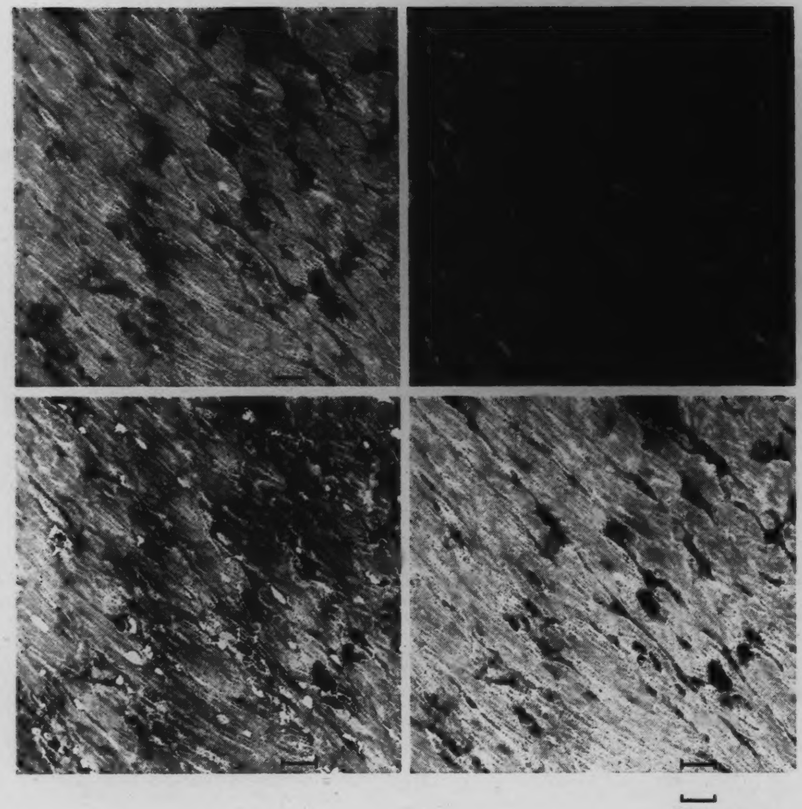

Figure 6.8 Co-localization of MMP-2 with TnI using immunofluorescent confocal microscopy, 20 μm scale bar. A fixed frozen section from a rat heart subjected to 20 min ischemia and 30 min reperfusion was immunostained using both anti-MMP-2 and anti-TnI antibodies. The nuclei were stained with DAPI Vectashield (blue). Upper left: Strong immunofluorescent signal for TnI (green) along the myofilaments is observed throughout myocytes. Upper right: MMP-2 immunofluorescence (red) is more heterogenous, however, both membrane and intracellular localization is evident. Bottom left: transmitted image of the section. Bottom right: co-localization image of MMP-2 with TnI shows an intermittent yet broadly distributed association pattern (yellow and yellow-orange).

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CHAPTER VII

CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

7.1 Conclusions

In the heart, I demonstrated a novel regulation pathway of MMP activity via oxidant stress. I also found that troponin I is an intracellular target of MMP-2 and its cleavage mediates at least in part, myocardial ischemia-reperfusion injury induced by MMP-2.

Oxidative stress causes cell dysfunction and eventually cell death when oxidants overwhelm the antioxidant defenses. Here I presented a novel mediator of oxidant-induced injury. In the heart exposed to exogenous peroxynitrite, there was a slow but progressive decline in cardiac mechanical function. I also found an increased release of MMP-2 into the coronary effluent which preceded the decline in cardiac mechanical function. The antioxidant glutathione prevented the increase in MMP-2 activity in the coronary effluent. Either glutathione or an inhibitor of MMPs, PD-166793, protected the heart from peroxynitrite-induced injury. In the normal heart, removal of basal NO generation in the coronary vasculature resulted in oxidative stress as measured by the level of lipid hydroperoxides. There was also an increase in MMP-2 activity in the coronary effluent of hearts treated with the NO synthase inhibitor L-NAME. Co-infusion of the NOS inhibitor with the NO donor SNAP not only prevented the increase in MMP-2 activity but also abolished the increase in lipid hydroperoxides. Taken together, these findings suggest that inhibition of MMP activity could be a novel strategy in the treatment of pathologies in which oxidative stress is involved. These studies also revealed a potential activation pathway of MMPs during myocardial ischemia-reperfusion injury. As mentioned in Chapter I, myocardial ischemia-reperfusion injury is considered to be an oxidative stress. The increase in MMP-2 activity may be a result of enhanced production of peroxynitrite during the first minute of reperfusion. In particular, the intracellular production of peroxynitrite, in the close vicinity of 72-kDa MMP-2, could activate it intracellularly via conformational change, without a loss of the propeptide domain.

MMP-2 plays a role in acute myocardial ischemia-reperfusion injury. I found that TIMP-4, the most abundant isoform of TIMP in the heart, was rapidly released during the first minute of reperfusion. Although there is also a simultaneous release of MMP-2 from the heart at this time, TIMP-4 apparently came predominantly from intracellular sources as shown by less sarcomere associated TIMP-4 in the thin myofilament fraction from hearts subjected to ischemia-reperfusion compared to aerobically perfused control hearts. Combined with the data in Chapter VI showing that there was an accumulation of MMP-2 in sarcomeres, despite its release into the coronary perfusate, it is evident that an imbalance between TIMPs and MMPs occurs during acute myocardial ischemia-reperfusion injury, perhaps even within the microenvironment of the thin myofilaments. An increase in tissue gelatinolytic activity was evident in the ischemic-reperfused heart, as shown by in situ zymography (Figure 5.6, Chapter V).

In looking for the molecular target of MMP-2, I also found that TnI, a regulatory element of contractile proteins, is susceptible to cleavage by MMP-2. TnI is proteolyzed by MMP-2 both in vitro and in the ex vivo intact heart. Inhibitors of MMPs could prevent the cleavage of TnI in both situations. The close association between TnI and MMP-2 was demonstrated by both immunoprecipitation, immunoelectron microscopy as well as confocal microscopy. These findings could be significant not only because they revealed an enzyme responsible for TnI cleavage but also disclosed that MMPs, contrary to the generally held belief, not only reside but also have an intracellular action during myocardial ischemia-reperfusion injury. Inhibition of MMPs may be a useful strategy in the treatment of myocardial ischemia-reperfusion injury. Figure 7.1 summarizes the model of MMP-induced myocardial injury during ischemia-reperfusion.

Figure 7.2 is a speculative illustration of what happens during myocardial ischemia-reperfusion. In normal aerobic hearts, inactive MMP-2 is expressed in endothelial cells, cardiac myocytes and other cardiac cells. Within the cardiac myocyte, the expression of MMP-2 is different between various organelles, with a low expression in myofilaments, if not at all. TIMP-4 is heavily expressed in cardiac myocytes and keeps net tissue MMP activity at a very low level. Immediately upon reperfusion following ischemia there is a rapid translocation and/or activation of MMP-2. There is a concurrent release of TIMP-4 from the cardiac myocytes as well. There is not only an accumulation of MMP-2 within the thin myofilaments, but also a release of MMP-2 into the coronary circulation from endothelial cells as well as cardiac myocytes. This is likely a means to limit the proteolytic damage caused by MMP-2. However extracellular MMP-2 may also play a role in setting vascular contractile tone or in the regulation of platelet aggregation. The enhanced intracellular MMP-2 activity and decreased TIMP level account for the increased net tissue MMP activity. MMP-2 induces injury to cardiac myocyte partially by cleavage of troponin I, as mentioned above.

The translocation mechanisms of MMP during ischemia-reperfusion remain an open question. Whether the translocation is an intercellular or intracellular process is unknown. Mammalian cells are equipped with sophisticated systems to not only move proteins out of them (secretion) but also to move them into cells. Based on the knowledge that many bacteria-derived toxins, which are Zn^{2+} endopeptidases, bind to unknown membrane receptors and enter into mammalian nerve terminals[1], I speculate that receptor-mediated endocytosis is a most likely scenario for the entry of MMP-2 into cells. The membrane associated proteins such as MT-MMP, integrins, α_2-macroglobulin receptor and possibly others may act as receptors for MMP. The MMP-receptor complex then undergoes internalization via a clathrin-dependent or –independent mechanism. Ubiquitination of MMP-2 may also be part of the process, knowing that not all ubiquitination leads to degradation. MMP itself may be a pore-forming protein which inserts into lipid bilayer membrane and facilitates the translocation of other MMP molecules.

The intracellular/interorganellular protein translocation is another fascinating subject[2]. The interorganellular protein translocation is a critical function in mammalian cells. For example, the mitochondrion has its own genome which only codes for approximately 13 proteins. All the other 1000 plus proteins in the mitochondria are imported from the cytosol. Mitochondria have developed a sophisticated transportation system to import proteins across outer membrane and inter membrane into its matrix. This system may well work to move MMP across membranes. Vesicular trafficking might also be the way to move MMP within cells.

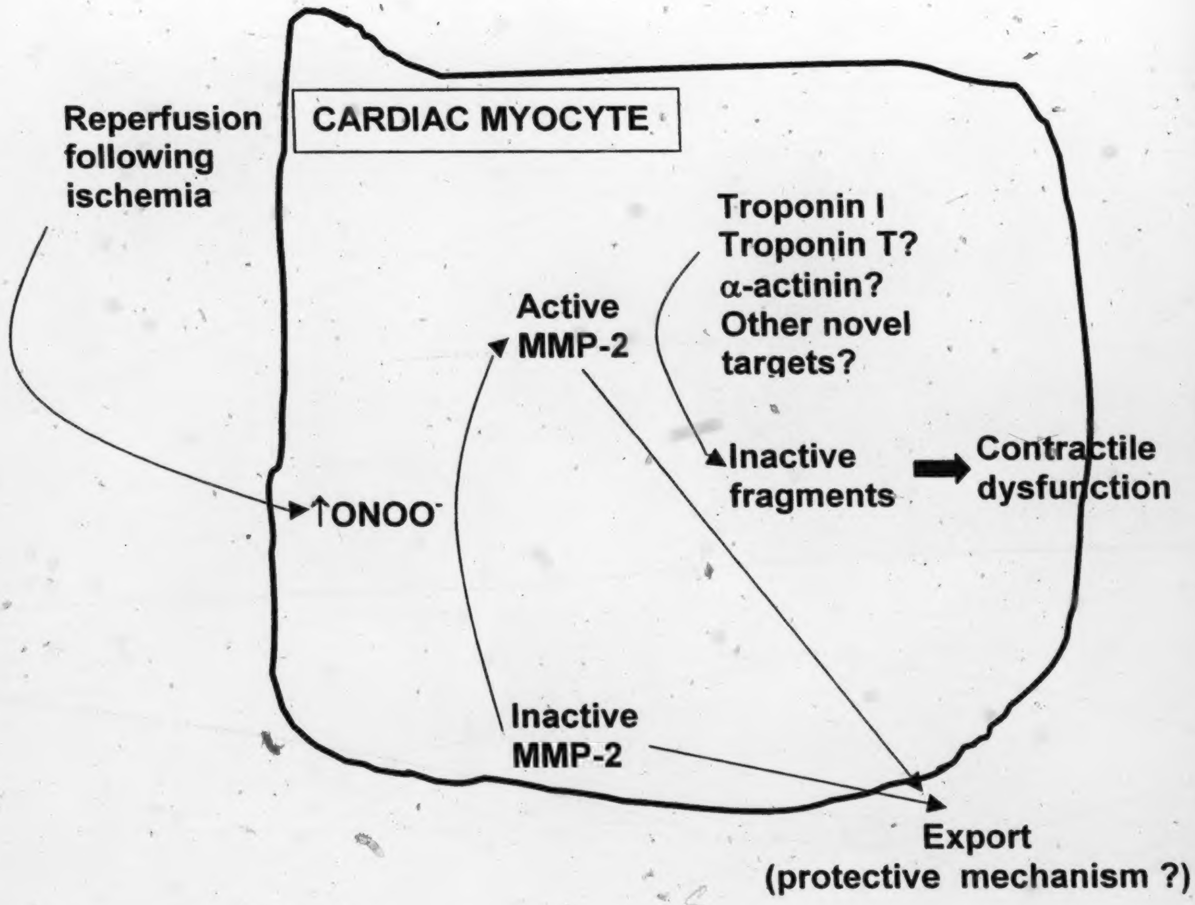
CARDIAC MYOCYTE
Reperfusion following ischemia
↑ONOO⁻
Active MMP-2
Troponin I
Troponin T?
α-actinin?
Other novel targets?
Inactive fragments
Contractile dysfunction
Inactive MMP-2
Export (protective mechanism ?)

Figure 7.1 Model of ischemia-reperfusion injury in the cardiac myocyte and the roles of $ONOO^-$, MMP-2, troponin I and other possible intracellular targets of MMP-2.

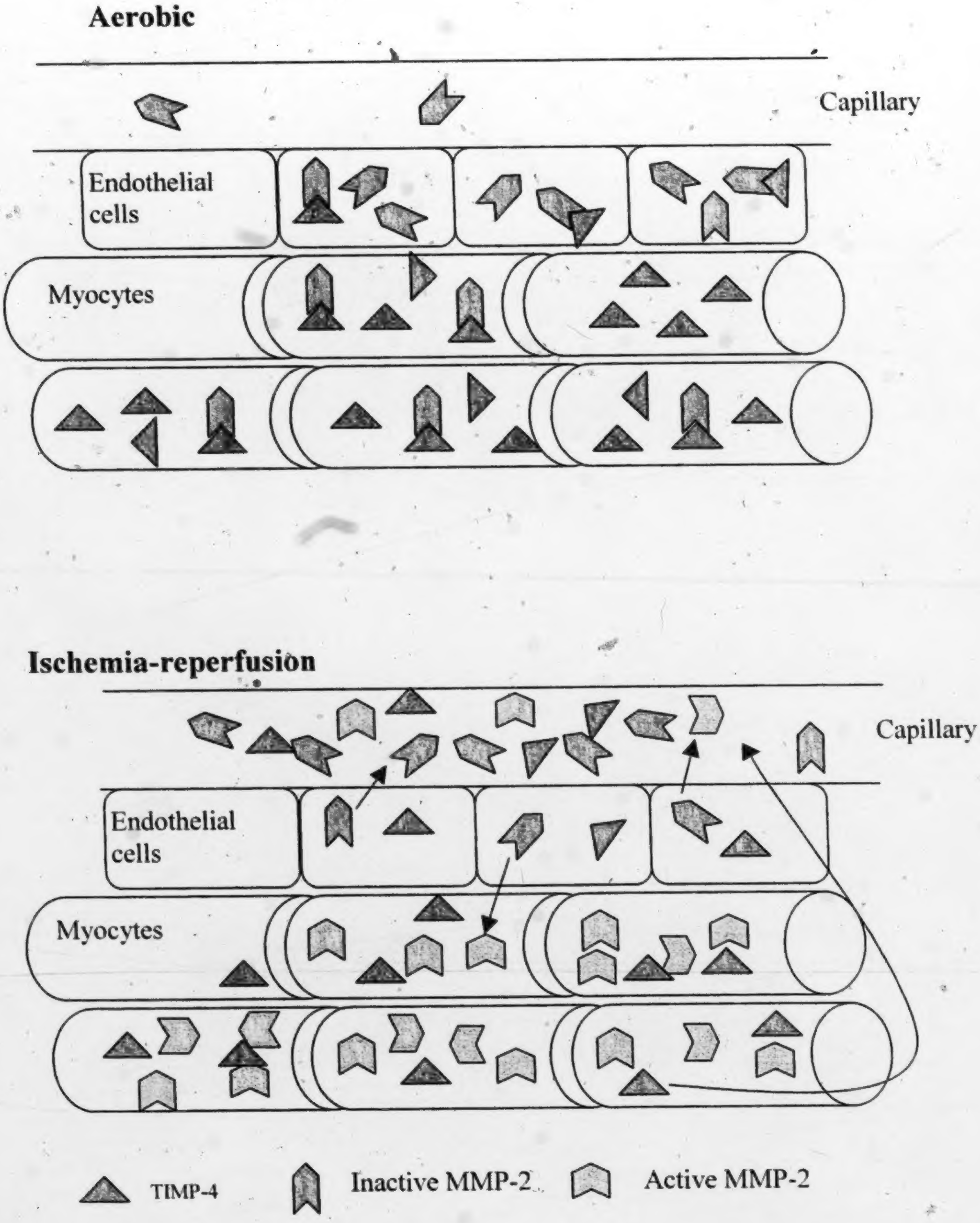
Aerobic
Capillary
Endothelial cells
Myocytes
Ischemia-reperfusion
Capillary
Endothelial cells
Myocytes
TIMP-4
Inactive MMP-2
Active MMP-2

Figure 7.2 Cardiac localization of MMP-2 and TIMP-4 under both normal aerobic perfusion and ischemia and reperfusion conditions.

7.2 Limitations

7.2.1 General limitations

Although the data presented in my thesis are compelling, caution should be exercised when extrapolating the findings to the in vivo situation and to their potential clinical utility. This is due to limitations in the experimental model. Isolated heart perfusion gives one the advantage of studying a particular drug treatment or intervention in a single organ without neurological or humoral influences. However, it is quite different from the normal physiological environment of the heart. Crystalloid perfusion solution is also very different from blood. It is evident that I did not observe any MMP-9 activity either in the coronary perfusate or the myocardium (Chapters III, IV, V and VI), reflecting the notion that MMP-9 is mainly produced by neutrophils[3], platelet[4] and macrophages[5] which are absent in crystalloid perfusion solution. Edema can occur in hearts perfused with crystalloid solutions. This could affect the release of MMPs. The perfusion pressure (60 mmHg in constant pressure mode with an average flow of 14 ml/min) and flow (10 ml/min in constant flow mode with an average pressure of 40 mmHg) are different from physiological pressure (mean arterial pressure 100 mmHg) and coronary flow (3 ml/min) in the rat. These parameters were suggested by Langendorff based on histological and metabolic factors[6]. The high in vitro coronary flow may cause more NO production than in vivo due to differences in shear stress [7]. It is possible that the role of basal NO production in the coronary vasculature is exaggerated in this study. As mentioned in Chapter I, isolated rat hearts aerobically perfused with crystalloid solution can only be kept stable for up to 2 hr. This short perfusion period does not allow one to find out whether the heart could fully recover from the cardiac mechanical depression seen as a result of ischemia-reperfusion. At least several hours are needed to allow the full recovery from cardiac depression caused by ischemia and reperfusion. Therefore, I cannot be completely sure whether the model used can completely reflect myocardial stunning.

Throughout the thesis I used gelatin zymography, a sensitive and well reproducible technique to detect MMP-2 and MMP-9 activities. However, a great deal of other MMPs which do not degrade gelatin efficiently, such as MMP-3, are not detected by gelatin zymography. In the present study I was therefore not able to find out the contribution and change in other MMPs and protease activities. All these limitations should be considered in future studies.

7.2.2 Limitations in Chapter III

I used the infusion of exogenous peroxynitrite to mimic the rapid myocardial production of peroxynitrite occurring during the first minute of reperfusion. Moreover, endogenous peroxynitrite is formed within endothelial cells, cardiac myocytes as well as in the microvascular lumen, which are in the vicinity of its intracellular targets. Peroxynitrite also has a very short half-life in a physiological environment such that relatively high concentrations of peroxynitrite were used in this study. Nevertheless, valuable information can still be obtained from this study when caution is exercised. In this study, only one concentration of the MMP inhibitor, PD-166793, was used due to its low solubility in its vehicle (0.05% DMSO).

7.2.3 *Limitations in Chapter V*

Although immunological studies identified the ~25-kDa protein associated with thin myofilaments as TIMP-4, only sequencing techniques would be able to identify it conclusively. Due to technical difficulties, such as the very low extraction rate of this protein from the heart, which did not allow me to have enough material for electron spray mass spectrometry, this could not be done in the present study. How the translocation of TIMP-4 from an intracellular location into the coronary perfusate occurs was also not explored. In situ zymography does not differentiate between the contribution of individual MMPs to the increased tissue gelatinolytic activity. A high concentration of EDTA (10 mM), which also could inhibit all Ca^{2+}/Zn^{2+}-dependent enzymes, was used to inhibit the tissue gelatinolytic activity during in situ zymography.

7.2.4 *Limitations in Chapter VI*

I used o-phenanthroline and doxycycline as pharmacological tools. Their broad inhibition spectrum of various MMPs does not allow me to pin-point the role of individual MMPs in the cleavage of troponin in the intact heart. These inhibitors, mainly acting as divalent ion chelators, may have other effects in the heart such as inhibition of other zinc or copper containing enzymes. Due to technical difficulties of getting enough protein from the isolated hearts for sequencing, there is no evidence to demonstrate that the cleavage of TnI in the ischemic-reperfused heart exactly matches what I saw in vitro. There is also no direct evidence whether the cleavage of TnI by MMP-2 happens in vivo.

7.3 Future directions

Many questions remain unresolved about the role of intracellular TIMP-4 within the cardiac myocyte. What are other possible biological actions of this intracellular protein? How does it translocate during myocardial ischemia-reperfusion? Does it behave differently from 23-kDa TIMP-4? The biological significance of the 25-kDa TIMP-4 inside the cardiac myocyte warrants further investigation. The sequence identity of this TIMP-4 is also required.

Although inhibition of MMP activity protects the heart from peroxynitrite-induced injury, the mechanism of MMP-mediated injury is not completely known. Whether proteolysis of TnI is part of the pathophysiology requires further study, particularly in vivo. One study has shown that the addition of exogenous oxidants to hearts subjected to ischemia-reperfusion resulted in a decrease in collagen content[8]. So the derangement in collagen structure could also be part of the mechanism of peroxynitrite-induced injury via MMPs.

I presented here clear-cut evidence showing that the proteolysis of the cardiac contractile protein regulatory element troponin I is part of the pathophysiology of myocardial ischemia-reperfusion injury mediated through MMP-2. New strategies should be used in future studies such as the MMP-2 deficient mouse in both in vitro and in vivo ischemia-reperfusion models, as well as the use of MMP-2 selective inhibitors. Moreover, other intracellular proteins could also be possible targets of MMPs including α-actitin, titin, desmin, sarcoplasmic reticulum Ca^{2+}-ATPase as well as troponin T, just to name a few.

The role of other MMPs should also be explored. As mentioned previously, several MMPs are expressed in the heart including MMP-1, 2, 3, 7, 9 and 13 (although not all of them are expressed in the rodent heart, for example, MMP-1 is not expressed in murine hearts). Using gelatin zymography only MMP-2 and MMP-9 are readily detectable. The roles of other MMPs are unclear. Other techniques such as casein zymography, western blot analysis and the Kunitz reaction to detect all proteases which are able to digest casein should be employed in the future in the order to find out the complete profile of proteases. 2-D gel zymography will be a very promising technique in fulfilling this requirement.

Another group of metalloproteinases are ADAMs, proteinases with a disintegrin- and metalloproteinase-domain, with 29 members to date[9]. In the heart, ADAM 9, 10, 12, 15 and 19 are expressed[10-12]. ADAMs linked to the cell membrane function in proteolysis, signaling, cell adhesion and fusion as well as in ectodomain shedding[9]. In the heart, ADAMs may also be of importance for structural remodeling of cardiac tissue by performing cleavage and secretion of surface-bound proteins. Whether they are involved in acute myocardial ischemia-reperfusion injury is not known.

This thesis also paved a road for many unique pharmacological approaches. For example, a compound with both a combination of an MMP inhibitory effect as well as an antioxidant action could be very useful in the treatment of many cardiac diseases such as heart failure and infarction. Lessons can be learned from tetracycline antibiotics due to their reported multiple pharmacological actions beyond their antibacterial effect, not only on collagenase activity but also as antioxidants. The idea of adding a NO donor moiety into the chemical structure of a MMP inhibitor could also be appealing. Indeed, an S-nitrosylated α1-protease inhibitor has been developed[13]. This compound can release NO, which inhibits platelet aggregation, and can also inhibit elastase. Whether such a similar compound which could release NO and inhibit MMPs could show therapeutic utility requires further study.

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